

biology methods. The appropriate nucleotides can be engineered in the cDNA to form convenient restriction sites and also allow the attachment of the protein cDNA to albumin cDNA similar to the method described for hGH. Also a targeting protein or peptide cDNA such as single chain antibody or peptides, such as nuclear localization signals, that can direct proteins inside the cells can be fused to the other end of albumin. The protein of interest and the targeting peptide is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA, or pC4:HSA which allows the fusion with albumin cDNA. In this manner both N- and C-terminal end of albumin are fused to other proteins. The fused cDNA is then excised from pPPC0005 and is inserted into a plasmid such as pSAC35 to allow the expression of the albumin fusion protein in yeast. All the above procedures can be performed using standard methods in molecular biology. The albumin fusion protein secreted from yeast can be collected and purified from the media and tested for its biological activity and its targeting activity using appropriate biochemical and biological tests.

Example 12: Preparation of HA-enzymes fusions

The cDNA for the enzyme of interest can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The enzyme cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA, or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 13: Bacterial Expression of an Albumin Fusion Protein

A polynucleotide encoding an albumin fusion protein of the present invention comprising a bacterial signal sequence is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' ends of the DNA sequence, to synthesize insertion fragments. The primers used to amplify the polynucleotide encoding insert should preferably contain

restriction sites, such as BamHI and XbaI, at the 5' end of the primers in order to clone the amplified product into the expression vector. For example, BamHI and XbaI correspond to the restriction enzyme sites on the bacterial expression vector pQE-9. (Qiagen, Inc., Chatsworth, CA). This plasmid vector encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter/operator (P/O), a ribosome binding site (RBS), a 6-histidine tag (6-His), and restriction enzyme cloning sites.

The pQE-9 vector is digested with BamHI and XbaI and the amplified fragment is ligated into the pQE-9 vector maintaining the reading frame initiated at the bacterial RBS. The ligation mixture is then used to transform the E. coli strain M15/rep4 (Qiagen, Inc.) which contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.⁶⁰⁰) of between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalactopyranoside) is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression.

Cells are grown for an extra 3 to 4 hours. Cells are then harvested by centrifugation (20 mins at 6000Xg). The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl or preferably in 8 M urea and concentrations greater than 0.14 M 2-mercaptoethanol by stirring for 3-4 hours at 4°C (see, e.g., Burton et al., Eur. J. Biochem. 179:379-387 (1989)).

The cell debris is removed by centrifugation, and the supernatant containing the polypeptide is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (available from QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the Ni-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist (1995) QIAGEN, Inc., *supra*).

Briefly, the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8. The column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the polypeptide is eluted with 6 M guanidine-HCl, pH 5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. Exemplary conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20

mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins are eluted by the addition of 250 mM imidazole. Imidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4° C or frozen at -80° C.

In addition to the above expression vector, the present invention further includes an expression vector, called pHE4a (ATCC Accession Number 209645, deposited on February 25, 1998) which contains phage operator and promoter elements operatively linked to a polynucleotide encoding an albumin fusion protein of the present invention, called pHE4a. (ATCC Accession Number 209645, deposited on February 25, 1998.) This vector contains: 1) a neomycinphosphotransferase gene as a selection marker, 2) an E. coli origin of replication, 3) a T5 phage promoter sequence, 4) two lac operator sequences, 5) a Shine-Delgarno sequence, and 6) the lactose operon repressor gene (lacIq). The origin of replication (oriC) is derived from pUC19 (LTI, Gaithersburg, MD). The promoter and operator sequences are made synthetically.

DNA can be inserted into the pHE4a by restricting the vector with NdeI and XbaI, BamHI, XhoI, or Asp718, running the restricted product on a gel, and isolating the larger fragment (the stuffer fragment should be about 310 base pairs). The DNA insert is generated according to PCR protocols described herein or otherwise known in the art, using PCR primers having restriction sites for NdeI (5' primer) and XbaI, BamHI, XhoI, or Asp718 (3' primer). The PCR insert is gel purified and restricted with compatible enzymes. The insert and vector are ligated according to standard protocols.

The engineered vector may be substituted in the above protocol to express protein in a bacterial system.

Example 14: Expression of an Albumin Fusion Protein in Mammalian Cells

The albumin fusion proteins of the present invention can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVII, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

Suitable expression vectors for use in practicing the present invention include, for example, vectors such as, pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), pCMVSPORT 2.0, and pCMVSPORT 3.0. Mammalian host cells that could be used include, but are not limited to, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the albumin fusion protein can be expressed in stable cell lines containing the polynucleotide encoding the albumin fusion protein integrated into a chromosome. The co-transfection with a selectable marker such as DHFR, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected polynucleotide encoding the fusion protein can also be amplified to express large amounts of the encoded fusion protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of interest. (See, e.g., Alt et al., J. Biol. Chem. 253:1357-1370 (1978); Hamlin et al., Biochem. et Biophys. Acta, 1097:107-143 (1990); Page et al., Biotechnology 9:64-68 (1991)). Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., Biochem J. 227:277-279 (1991); Bebbington et al., Bio/Technology 10:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

Derivatives of the plasmid pSV2-dhfr (ATCC Accession No. 37146), the expression vectors pC4 (ATCC Accession No. 209646) and pC6 (ATCC Accession No. 209647) contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., Cell 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors also contain the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter.

Specifically, the plasmid pC6, for example, is digested with appropriate restriction enzymes and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

A polynucleotide encoding an albumin fusion protein of the present invention is generated using techniques known in the art and this polynucleotide is amplified using PCR technology known in the art. If a naturally occurring signal sequence is used to produce the fusion protein of the present invention, the vector does not need a second signal peptide.

Alternatively, if a naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International Publication No. WO 96/34891.)

5 The amplified fragment encoding the fusion protein of the invention is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

10 The amplified fragment encoding the albumin fusion protein of the invention is then digested with the same restriction enzyme and purified on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene is used for transfection. Five μg of the expression plasmid pC6 or pC4 is cotransfected with 0.5 μg of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM , 2 μM , 5 μM , 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μM . Expression of the desired fusion protein is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Example 15: Multifusion Fusions

30 The albumin fusion proteins (e.g., containing a Therapeutic protein (or fragment or variant thereof) fused to albumin (or a fragment or variant thereof)) may additionally be fused to other proteins to generate "multifusion proteins". These multifusion proteins can be used for a variety of applications. For example, fusion of the albumin fusion proteins of the invention to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See e.g., EP A 394,827; Traunecker et al., Nature 331:84-86 (1988)). Nuclear localization signals fused to the polypeptides of the present invention can target the protein to

a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of an albumin fusion protein. Furthermore, the fusion of additional protein sequences to the albumin fusion proteins of the invention may further increase the solubility and/or stability of the fusion protein. The fusion proteins described above can be made using or routinely modifying techniques known in the art and/or by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian or yeast expression vector.

For example, if pC4 (ATCC Accession No. 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and a polynucleotide encoding an albumin fusion protein of the present invention (generated and isolated using techniques known in the art), is ligated into this BamHI site. Note that the polynucleotide encoding the fusion protein of the invention is cloned without a stop codon, otherwise a Fc containing fusion protein will not be produced.

If the naturally occurring signal sequence is used to produce the albumin fusion protein of the present invention, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International Publication No. WO 96/34891.)

Human IgG Fc region:

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GGGATCCGGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGC
25 CCAGCACCTGAATTCGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCAAAACCCA
AGGACACCCTCATGATCTCCCGGACTCCTGAGGTCACATGCGTGGTGGTGGACG
TAAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGG
TGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTG
TGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACA
30 AGTGCAAGGTCTCCAACAAAGCCCTCCCAACCCCCATCGAGAAAACCATCTCCAA
AGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGA
TGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCA
AGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACACTACAA
GACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC
35 ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATG
CATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA

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AATGAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO: 36)

Example 16: Production of an Antibody from an Albumin Fusion Protein

5 a) Hybridoma Technology

Antibodies that bind the albumin fusion proteins of the present invention and portions of the albumin fusion proteins of the present invention (e.g., the Therapeutic protein portion or albumin portion of the fusion protein) can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, a preparation of an albumin fusion protein of the invention or a portion of an albumin fusion protein of the invention is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

Monoclonal antibodies specific for an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, are prepared using hybridoma technology (Kohler et al., Nature 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, an animal (preferably a mouse) is immunized with an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention.

Alternatively, additional antibodies capable of binding to an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the an albumin fusion protein of the

invention (or portion of an albumin fusion protein of the invention) -specific antibody can be blocked by the fusion protein of the invention, or a portion of an albumin fusion protein of the invention. Such antibodies comprise anti-idiotypic antibodies to the fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibody and are used to immunize an animal to induce formation of further fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibodies.

For *in vivo* use of antibodies in humans, an antibody is "humanized". Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric and humanized antibodies are known in the art and are discussed herein. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., International Publication No. WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985)).

b) Isolation Of Antibody Fragments Directed Against an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention From A Library Of scFvs

Naturally occurring V-genes isolated from human PBLs are constructed into a library of antibody fragments which contain reactivities against an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein by reference in its entirety).

Rescue of the Library. A library of scFvs is constructed from the RNA of human PBLs as described in International Publication No. WO 92/01047. To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 μ g/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2×10^8 TU of delta gene 3 helper (M13 delta gene III, see International Publication No. WO 92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin and grown overnight. Phage are prepared as described in International Publication No. WO 92/01047.

M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater

avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8,400
5 r.p.m. for 10 min), resuspended in 300 ml 2xTY broth containing 100 µg ampicillin/ml and 25 µg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 µm filter (Minisart NML; Sartorius) to give a final concentration of approximately 10¹³ transducing
10 units/ml (ampicillin-resistant clones).

Panning of the Library. Immuntubes (Nunc) are coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 10¹³ TU of phage is applied
15 to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to
20 infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37°C. The E. coli are then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20
25 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of Binders. Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of an albumin fusion protein of the invention, or a portion of an albumin fusion protein
30 of the invention, in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., International Publication No. WO 92/01047) and then by sequencing. These ELISA positive clones may also be further characterized by techniques known in the art, such as, for example, epitope mapping, binding affinity, receptor signal transduction, ability to block or competitively inhibit antibody/antigen binding,
35 and competitive agonistic or antagonistic activity.

Example 17: Method of Treatment Using Gene Therapy-Ex Vivo

One method of gene therapy transplants fibroblasts, which are capable of expressing an albumin fusion protein of the present invention, onto a patient. Generally, fibroblasts are
5 obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of
10 the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer
15 is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

20 Polynucleotides encoding an albumin fusion protein of the invention can be generated using techniques known in the art amplified using PCR primers which correspond to the 5' and 3' end sequences and optionally having appropriate restriction sites and initiation/stop codons, if necessary. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear
25 backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector has the gene of interest properly inserted.

30 The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as
35 producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is

harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether the albumin fusion protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

Example 18: Method of Treatment Using Gene Therapy - In Vivo

Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences encoding an albumin fusion protein of the invention into an animal. Polynucleotides encoding albumin fusion proteins of the present invention may be operatively linked to (i.e., associated with) a promoter or any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata et al., Cardiovasc. Res. 35(3):470-479 (1997); Chao et al., Pharmacol. Res. 35(6):517-522 (1997); Wolff, Neuromuscul. Disord. 7(5):314-318 (1997); Schwartz et al., Gene Ther. 3(5):405-411 (1996); Tsurumi et al., Circulation 94(12):3281-3290 (1996) (incorporated herein by reference).

The polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, polynucleotides encoding albumin fusion proteins of the present invention may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al. (1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

The polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The polynucleotide construct can be delivered to the interstitial space of tissues within an animal, including muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected polynucleotide in muscle *in vivo* is determined

as follows. Suitable template DNA for production of mRNA coding for polypeptide of the present invention is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for protein expression. A time course for fusion protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be used to extrapolate proper dosages and other treatment parameters in humans and other animals using naked DNA.

Example 19: Transgenic Animals

The albumin fusion proteins of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express fusion proteins of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the polynucleotides encoding the albumin fusion proteins of the invention into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell

56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pluripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides encoding albumin fusion proteins of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

The present invention provides for transgenic animals that carry the polynucleotides encoding the albumin fusion proteins of the invention in all their cells, as well as animals which carry these polynucleotides in some, but not all their cells, *i.e.*, mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)).

The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide encoding the fusion protein of the invention be integrated into the chromosomal site of the endogenous gene corresponding to the Therapeutic protein portion or albumin portion of the fusion protein of the invention, gene targeting is preferred.

Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the polynucleotide encoding the fusion protein of the invention has taken place. The level

of mRNA expression of the polynucleotide encoding the fusion protein of the invention in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of fusion protein-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the fusion protein.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene (i.e., polynucleotide encoding an albumin fusion protein of the invention) on a distinct background that is appropriate for an experimental model of interest.

Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of fusion proteins of the invention and the Therapeutic protein and/or albumin component of the fusion protein of the invention, studying conditions and/or disorders associated with aberrant expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

Example 20: Assays Detecting Stimulation or Inhibition of B cell Proliferation and Differentiation

Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B cell responsiveness including IL-2, IL-4, IL-5, IL-6, IL-7, IL10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce activation, proliferation, differentiation, homing, tolerance and death among B cell populations.

One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands

CD154, CD70, and CD153 have been found to regulate a variety of immune responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of B-cell populations and their precursors.

In Vitro Assay- Albumin fusion proteins of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin) can be assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of an albumin fusion protein of the invention on purified human tonsillar B cells, measured qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B cells are cultured in the presence of either formalin-fixed *Staphylococcus aureus* Cowan I (SAC) or immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B cells by magnetic bead (MACS) depletion of CD3-positive cells. The resulting cell population is greater than 95% B cells as assessed by expression of CD45R(B220).

Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10^5 B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5×10^{-5} M 2ME, 100U/ml penicillin, 10ug/ml streptomycin, and 10^{-5} dilution of SAC) in a total volume of 150ul. Proliferation or inhibition is quantitated by a 20h pulse (1uCi/well) with 3 H-thymidine (6.7 Ci/mM) beginning 72h post factor addition. The positive and negative controls are IL2 and medium respectively.

In vivo Assay- BALB/c mice are injected (i.p.) twice per day with buffer only, or 2 mg/Kg of an albumin fusion protein of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin). Mice receive this treatment for 4 consecutive days, at which time they are sacrificed and various tissues and serum collected for analyses. Comparison of H&E sections from normal spleens and spleens treated with the albumin fusion protein of the invention identify the results of the activity of the fusion protein on spleen cells, such as the diffusion of peri-arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-

cell populations. Immunohistochemical studies using a B cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell representation within loosely defined B-cell zones that infiltrate established T-cell regions.

5 Flow cytometric analyses of the spleens from mice treated with the albumin fusion protein is used to indicate whether the albumin fusion protein specifically increases the proportion of ThB+, CD45R(B220)dull B cells over that which is observed in control mice.

Likewise, a predicted consequence of increased mature B-cell representation *in vivo* is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared
10 between buffer and fusion protein treated mice.

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

15 ***Example 21: T Cell Proliferation Assay***

A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of ³H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 µl/well of mAb to CD3 (HIT3a, Pharmingen) or isotype-matched control mAb (B33.1) overnight at 4 degrees C (1 µg/ml in .05M bicarbonate buffer, pH 9.5), then
20 washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10⁴/well) of mAb coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of an albumin fusion protein of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin) (total
25 volume 200 µl). Relevant protein buffer and medium alone are controls. After 48 hr. culture at 37 degrees C, plates are spun for 2 min. at 1000 rpm and 100 µl of supernatant is removed and stored -20 degrees C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 µl of medium containing 0.5 uCi of ³H-thymidine and cultured at 37 degrees C for 18-24 hr. Wells are harvested and
30 incorporation of ³H-thymidine used as a measure of proliferation. Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances proliferation. Control antibody which does not induce proliferation of T cells is used as the negative control for the effects of fusion proteins of the invention.

The studies described in this example tested activity of fusion proteins of the
35 invention. However, one skilled in the art could easily modify the exemplified studies to test

the activity of fusion proteins or polynucleotides of the invention (e.g., gene therapy).

Example 22: Effect of Fusion Proteins of the Invention on the Expression of MHC Class II, Costimulatory and Adhesion Molecules and Cell Differentiation of Monocytes and Monocyte-Derived Human Dendritic Cells

Dendritic cells are generated by the expansion of proliferating precursors found in the peripheral blood: adherent PBMC or elutriated monocytic fractions are cultured for 7-10 days with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). These dendritic cells have the characteristic phenotype of immature cells (expression of CD1, CD80, CD86, CD40 and MHC class II antigens). Treatment with activating factors, such as TNF- α , causes a rapid change in surface phenotype (increased expression of MHC class I and II, costimulatory and adhesion molecules, downregulation of FC γ RII, upregulation of CD83). These changes correlate with increased antigen-presenting capacity and with functional maturation of the dendritic cells.

FACS analysis of surface antigens is performed as follows. Cells are treated 1-3 days with increasing concentrations of an albumin fusion protein of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Effect on the production of cytokines. Cytokines generated by dendritic cells, in particular IL-12, are important in the initiation of T-cell dependent immune responses. IL-12 strongly influences the development of Th1 helper T-cell immune response, and induces cytotoxic T and NK cell function. An ELISA is used to measure the IL-12 release as follows.

Dendritic cells (10^6 /ml) are treated with increasing concentrations of an albumin fusion protein of the invention for 24 hours. LPS (100 ng/ml) is added to the cell culture as positive control. Supernatants from the cell cultures are then collected and analyzed for IL-12 content using commercial ELISA kit (e.g., R & D Systems (Minneapolis, MN)). The standard protocols provided with the kits are used.

Effect on the expression of MHC Class II, costimulatory and adhesion molecules.

Three major families of cell surface antigens can be identified on monocytes: adhesion molecules, molecules involved in antigen presentation, and Fc receptor. Modulation of the expression of MHC class II antigens and other costimulatory molecules, such as B7 and ICAM-1, may result in changes in the antigen presenting capacity of monocytes and ability to

induce T cell activation. Increased expression of Fc receptors may correlate with improved monocyte cytotoxic activity, cytokine release and phagocytosis.

FACS analysis is used to examine the surface antigens as follows. Monocytes are treated 1-5 days with increasing concentrations of an albumin fusion protein of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Monocyte activation and/or increased survival. Assays for molecules that activate (or alternatively, inactivate) monocytes and/or increase monocyte survival (or alternatively, decrease monocyte survival) are known in the art and may routinely be applied to determine whether a molecule of the invention functions as an inhibitor or activator of monocytes. Albumin fusion proteins of the invention can be screened using the three assays described below. For each of these assays, Peripheral blood mononuclear cells (PBMC) are purified from single donor leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes are isolated from PBMC by counterflow centrifugal elutriation.

Monocyte Survival Assay. Human peripheral blood monocytes progressively lose viability when cultured in absence of serum or other stimuli. Their death results from internally regulated processes (apoptosis). Addition to the culture of activating factors, such as TNF-alpha dramatically improves cell survival and prevents DNA fragmentation. Propidium iodide (PI) staining is used to measure apoptosis as follows. Monocytes are cultured for 48 hours in polypropylene tubes in serum-free medium (positive control), in the presence of 100 ng/ml TNF-alpha (negative control), and in the presence of varying concentrations of the fusion protein to be tested. Cells are suspended at a concentration of 2×10^6 /ml in PBS containing PI at a final concentration of 5 μ g/ml, and then incubated at room temperature for 5 minutes before FACScan analysis. PI uptake has been demonstrated to correlate with DNA fragmentation in this experimental paradigm.

Effect on cytokine release. An important function of monocytes/macrophages is their regulatory activity on other cellular populations of the immune system through the release of cytokines after stimulation. An ELISA to measure cytokine release is performed as follows. Human monocytes are incubated at a density of 5×10^5 cells/ml with increasing concentrations of an albumin fusion protein of the invention and under the same conditions, but in the

absence of the fusion protein. For IL-12 production, the cells are primed overnight with IFN (100 U/ml) in the presence of the fusion protein. LPS (10 ng/ml) is then added. Conditioned media are collected after 24h and kept frozen until use. Measurement of TNF-alpha, IL-10, MCP-1 and IL-8 is then performed using a commercially available ELISA kit (e.g., R & D Systems (Minneapolis, MN)) and applying the standard protocols provided with the kit.

Oxidative burst. Purified monocytes are plated in 96-w plate at 2×10^5 cell/well. Increasing concentrations of an albumin fusion protein of the invention are added to the wells in a total volume of 0.2 ml culture medium (RPMI 1640 + 10% FCS, glutamine and antibiotics). After 3 days incubation, the plates are centrifuged and the medium is removed from the wells. To the macrophage monolayers, 0.2 ml per well of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of HRPO) is added, together with the stimulant (200 nM PMA). The plates are incubated at 37°C for 2 hours and the reaction is stopped by adding 20 μ l 1N NaOH per well. The absorbance is read at 610 nm. To calculate the amount of H_2O_2 produced by the macrophages, a standard curve of a H_2O_2 solution of known molarity is performed for each experiment.

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins or polynucleotides of the invention (e.g., gene therapy).

Example 23: Biological Effects of Fusion Proteins of the Invention

Astrocyte and Neuronal Assays

Albumin fusion proteins of the invention can be tested for activity in promoting the survival, neurite outgrowth, or phenotypic differentiation of cortical neuronal cells and for inducing the proliferation of glial fibrillary acidic protein immunopositive cells, astrocytes. The selection of cortical cells for the bioassay is based on the prevalent expression of FGF-1 and FGF-2 in cortical structures and on the previously reported enhancement of cortical neuronal survival resulting from FGF-2 treatment. A thymidine incorporation assay, for example, can be used to elucidate an albumin fusion protein of the invention's activity on these cells.

Moreover, previous reports describing the biological effects of FGF-2 (basic FGF) on cortical or hippocampal neurons *in vitro* have demonstrated increases in both neuron survival and neurite outgrowth (Walicke et al., "Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension." *Proc. Natl. Acad. Sci.*

USA 83:3012-3016. (1986), assay herein incorporated by reference in its entirety). However, reports from experiments done on PC-12 cells suggest that these two responses are not necessarily synonymous and may depend on not only which FGF is being tested but also on which receptor(s) are expressed on the target cells. Using the primary cortical neuronal culture paradigm, the ability of an albumin fusion protein of the invention to induce neurite outgrowth can be compared to the response achieved with FGF-2 using, for example, a thymidine incorporation assay.

Fibroblast and endothelial cell assays

Human lung fibroblasts are obtained from Clonetics (San Diego, CA) and maintained in growth media from Clonetics. Dermal microvascular endothelial cells are obtained from Cell Applications (San Diego, CA). For proliferation assays, the human lung fibroblasts and dermal microvascular endothelial cells can be cultured at 5,000 cells/well in a 96-well plate for one day in growth medium. The cells are then incubated for one day in 0.1% BSA basal medium. After replacing the medium with fresh 0.1% BSA medium, the cells are incubated with the test fusion protein of the invention proteins for 3 days. Alamar Blue (Alamar Biosciences, Sacramento, CA) is added to each well to a final concentration of 10%. The cells are incubated for 4 hr. Cell viability is measured by reading in a CytoFluor fluorescence reader. For the PGE₂ assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or fusion protein of the invention with or without IL-1 α for 24 hours. The supernatants are collected and assayed for PGE₂ by EIA kit (Cayman, Ann Arbor, MI). For the IL-6 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or with or without an albumin fusion protein of the invention and/or IL-1 α for 24 hours. The supernatants are collected and assayed for IL-6 by ELISA kit (Endogen, Cambridge, MA).

Human lung fibroblasts are cultured with FGF-2 or an albumin fusion protein of the invention for 3 days in basal medium before the addition of Alamar Blue to assess effects on growth of the fibroblasts. FGF-2 should show a stimulation at 10 - 2500 ng/ml which can be used to compare stimulation with the fusion protein of the invention.

Cell proliferation based on [3H]thymidine incorporation

The following [3H]Thymidine incorporation assay can be used to measure the effect

of a Therapeutic proteins, e.g., growth factor proteins, on the proliferation of cells such as fibroblast cells, epithelial cells or immature muscle cells.

Sub-confluent cultures are arrested in G1 phase by an 18 h incubation in serum-free medium. Therapeutic proteins are then added for 24 h and during the last 4 h, the cultures are
5 labeled with [3H]thymidine, at a final concentration of 0.33 μ M (25 Ci/mmol, Amersham, Arlington Heights, IL). The incorporated [3H]thymidine is precipitated with ice-cold 10% trichloroacetic acid for 24 h. Subsequently, the cells are rinsed sequentially with ice-cold 10% trichloroacetic acid and then with ice-cold water. Following lysis in 0.5 M NaOH, the lysates and PBS rinses (500 ml) are pooled, and the amount of radioactivity is measured.

10

Parkinson Models.

The loss of motor function in Parkinson's disease is attributed to a deficiency of striatal dopamine resulting from the degeneration of the nigrostriatal dopaminergic projection neurons. An animal model for Parkinson's that has been extensively characterized involves
15 the systemic administration of 1-methyl-4 phenyl 1,2,3,6-tetrahydropyridine (MPTP). In the CNS, MPTP is taken-up by astrocytes and catabolized by monoamine oxidase B to 1-methyl-4-phenyl pyridine (MPP⁺) and released. Subsequently, MPP⁺ is actively accumulated in dopaminergic neurons by the high-affinity reuptake transporter for dopamine. MPP⁺ is then concentrated in mitochondria by the electrochemical gradient and selectively inhibits
20 nicotinamide adenine disphosphate: ubiquinone oxidoreductionase (complex I), thereby interfering with electron transport and eventually generating oxygen radicals.

It has been demonstrated in tissue culture paradigms that FGF-2 (basic FGF) has trophic activity towards nigral dopaminergic neurons (Ferrari et al., Dev. Biol. 1989). Recently, Dr. Unsicker's group has demonstrated that administering FGF-2 in gel foam
25 implants in the striatum results in the near complete protection of nigral dopaminergic neurons from the toxicity associated with MPTP exposure (Otto and Unsicker, J. Neuroscience, 1990).

Based on the data with FGF-2, an albumin fusion protein of the invention can be evaluated to determine whether it has an action similar to that of FGF-2 in enhancing
30 dopaminergic neuronal survival *in vitro* and it can also be tested *in vivo* for protection of dopaminergic neurons in the striatum from the damage associated with MPTP treatment. The potential effect of an albumin fusion protein of the invention is first examined *in vitro* in a dopaminergic neuronal cell culture paradigm. The cultures are prepared by dissecting the midbrain floor plate from gestation day 14 Wistar rat embryos. The tissue is dissociated with
35 trypsin and seeded at a density of 200,000 cells/cm² on polyorthinine-laminin coated glass coverslips. The cells are maintained in Dulbecco's Modified Eagle's medium and F12 medium

containing hormonal supplements (N1). The cultures are fixed with paraformaldehyde after 8 days in vitro and are processed for tyrosine hydroxylase, a specific marker for dopaminergic neurons, immunohistochemical staining. Dissociated cell cultures are prepared from embryonic rats. The culture medium is changed every third day and the factors are also added at that time.

Since the dopaminergic neurons are isolated from animals at gestation day 14, a developmental time which is past the stage when the dopaminergic precursor cells are proliferating, an increase in the number of tyrosine hydroxylase immunopositive neurons would represent an increase in the number of dopaminergic neurons surviving *in vitro*. Therefore, if a Therapeutic protein acts to prolong the survival of dopaminergic neurons, it would suggest that the fusion protein may be involved in Parkinson's Disease.

The studies described in this example tested activity of albumin fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 24: The Effect of Albumin Fusion Proteins of the Invention on the Growth of Vascular Endothelial Cells

On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at $2-5 \times 10^4$ cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin. An albumin fusion protein of the invention, and positive controls, such as VEGF and basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

An increase in the number of HUVEC cells indicates that the fusion protein may proliferate vascular endothelial cells, while a decrease in the number of HUVEC cells indicates that the fusion protein inhibits vascular endothelial cells.

The studies described in this example tested activity of an albumin fusion protein of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of a fusion protein and polynucleotides of the invention.

Example 25: Rat Corneal Wound Healing Model

This animal model shows the effect of an albumin fusion protein of the invention on neovascularization. The experimental protocol includes:

- Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.
- Inserting a spatula below the lip of the incision facing the outer corner of the eye.

Making a pocket (its base is 1-1.5 mm from the edge of the eye).

Positioning a pellet, containing 50ng- 5ug of an albumin fusion protein of the invention, within the pocket.

5 Treatment with an an albumin fusion protein of the invention can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

The studies described in this example test the activity of an albumin fusion protein of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

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Example 26: Diabetic Mouse and Glucocorticoid-Impaired

Wound Healing Models

Diabetic db+/db+ Mouse Model.

15 To demonstrate that an albumin fusion protein of the invention accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al.*, *J. Surg. Res.* 52:389 (1992); Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235
20 (1990)).

The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/-) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al.* *Proc. Natl. Acad. Sci. USA* 25 77:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al.*, *J. Immunol.* 120:1375 (1978); Debray-Sachs, M. *et al.*, *Clin. Exp. Immunol.* 51(1):1-7 (1983); Leiter *et al.*, *Am. J. of Pathol.* 114:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular
30 lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al.*, *Exp. Neurol.* 83(2):221-232 (1984); Robertson *et al.*, *Diabetes* 29(1):60-67 (1980); Giacomelli *et al.*, *Lab Invest.* 40(4):460-473 (1979); Coleman, D.L., *Diabetes* 31 (Suppl):1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al.*,
35 *J. Immunol.* 120:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may

be similar to the healing observed in human diabetes (Greenhalgh, *et al.*, *Am. J. of Pathol.* 136:1235-1246 (1990)).

Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med.* 172:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

An albumin fusion protein of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) untreated group, and 3) treated group.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by

establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm², the corresponding size of the dermal punch. Calculations are made using the following formula:

$$5 \quad \frac{[\text{Open area on day 8}] - [\text{Open area on day 1}]}{[\text{Open area on day 1}]}$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with an albumin fusion protein of the invention. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

30

Steroid Impaired Rat Model

The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahlet *et al.*, *J. Immunol.* 115: 476-481 (1975); Werb *et al.*, *J. Exp. Med.* 147:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert *et al.*, *An.*

Intern. Med. 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck *et al.*, *Growth Factors*. 5: 295-304 (1991); Haynes *et al.*, *J. Clin. Invest.* 61: 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In: Antiinflammatory Steroid Action: Basic and Clinical Aspects*, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck *et al.*, *Growth Factors*. 5: 295-304 (1991); Haynes *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In: Antiinflammatory Steroid Action: Basic and Clinical Aspects*, Academic Press, New York, pp. 280-302 (1989); Pierce *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 2229-2233 (1989)).

To demonstrate that an albumin fusion protein of the invention can accelerate the healing process, the effects of multiple topical applications of the fusion protein on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

The wounding protocol is followed according to that described above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine-(50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The fusion protein of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

5 Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Three groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated: 1) Untreated group 2) Vehicle placebo control 3) treated groups.

10 Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm², the corresponding size of the dermal punch. Calculations are made using the following formula:

15

$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with an albumin fusion protein of the invention. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

20 Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

The studies described in this example tested activity of an albumin fusion protein of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

30

Example 27: Lymphedema Animal Model

The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of an albumin fusion protein of the invention in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and

35

histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosus and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect of plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

Circumference Measurements: Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people and those 2 readings are averaged. Readings are taken

from both control and edematous limbs.

Volumetric Measurements: On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), and both legs are shaved and
5 equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and Ca^{2+} comparison.

10 Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs are amputated using a quillitine, then both experimental and control legs are cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

Histological Preparations: The transverse muscle located behind the knee (popliteal)
15 area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle is observed under fluorescent microscopy for lymphatics..

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test
20 the activity of fusion protein and polynucleotides of the invention (e.g., gene therapy).

Example 28: Suppression of TNF alpha-Induced Adhesion Molecule Expression by an Albumin Fusion Protein of the Invention

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves
25 specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The
30 expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

Tumor necrosis factor alpha (TNF-a), a potent proinflammatory cytokine, is a
35 stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

The potential of an albumin fusion protein of the invention to mediate a suppression of TNF- α induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM expression on TNF- α treated ECs when co-stimulated with a member of the FGF family of proteins.

5 To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2; Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO₂. HUVECs are seeded in 96-well plates at concentrations of 1×10^4 cells/well in EGM medium at 37 degree C for 18-24 hrs or until
10 confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given cytokine and/or growth factor(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well
15 plate to confluence. Growth medium is removed from the cells and replaced with 90 μ l of 199 Medium (10% FBS). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10 μ l volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μ l of 0.1% paraformaldehyde-PBS(with Ca⁺⁺ and Mg⁺⁺) is added
20 to each well. Plates are held at 4°C for 30 min.

Fixative is then removed from the wells and wells are washed 1X with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10 μ l of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μ g/ml (1:10 dilution of 0.1 mg/ml
25 stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA.

Then add 20 μ l of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH
30 10.4). 100 μ l of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphatase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μ l of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μ l of pNPP reagent must then be added to each of the standard wells. The plate must be
35 incubated at 37°C for 4h. A volume of 50 μ l of 3M NaOH is added to all wells. The results are quantified on a plate reader at 405 nm. The background subtraction option is used on

blank wells filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

5 The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 29: Construction of GAS Reporter Construct

10 One signal transduction pathway involved in the differentiation and proliferation of cells is called the Jaks-STATs pathway. Activated proteins in the Jaks-STATs pathway bind to gamma activation site "GAS" elements or interferon-sensitive responsive element ("ISRE"), located in the promoter of many genes. The binding of a protein to these elements alter the expression of the associated gene.

15 GAS and ISRE elements are recognized by a class of transcription factors called Signal Transducers and Activators of Transcription, or "STATs." There are six members of the STATs family. Stat1 and Stat3 are present in many cell types, as is Stat2 (as response to IFN-alpha is widespread). Stat4 is more restricted and is not in many cell types though it has been found in T helper class I, cells after treatment with IL-12. Stat5 was originally called mammary growth factor, but has been found at higher concentrations in other cells including
20 myeloid cells. It can be activated in tissue culture cells by many cytokines.

The STATs are activated to translocate from the cytoplasm to the nucleus upon tyrosine phosphorylation by a set of kinases known as the Janus Kinase ("Jaks") family. Jaks represent a distinct family of soluble tyrosine kinases and include Tyk2, Jak1, Jak2, and Jak3. These kinases display significant sequence similarity and are generally catalytically
25 inactive in resting cells.

The Jaks are activated by a wide range of receptors summarized in the Table below. (Adapted from review by Schidler and Darnell, Ann. Rev. Biochem. 64:621-51 (1995)). A cytokine receptor family, capable of activating Jaks, is divided into two groups: (a) Class 1 includes receptors for IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15, Epo, PRL, GH, G-CSF, GM-CSF, LIF, CNTF, and thrombopoietin; and (b) Class 2 includes IFN-a, IFN-g, and IL-10. The Class 1 receptors share a conserved cysteine motif (a set of four conserved cysteines and one tryptophan) and a WSXWS motif (a membrane proximal region encoding Trp-Ser-Xaa-Trp-Ser (SEQ ID NO: 37)).
30

Thus, on binding of a ligand to a receptor, Jaks are activated, which in turn activate
35 STATs, which then translocate and bind to GAS elements. This entire process is encompassed in the Jaks-STATs signal transduction pathway. Therefore, activation of the

Jaks-STATs pathway, reflected by the binding of the GAS or the ISRE element, can be used to indicate proteins involved in the proliferation and differentiation of cells. For example, growth factors and cytokines are known to activate the Jaks-STATs pathway (See Table below). Thus, by using GAS elements linked to reporter molecules, activators of the Jaks-STATs pathway can be identified.

5

<u>Ligand</u>	<u>JAKs</u> <u>tyk2</u>	<u>Jak1</u>	<u>Jak2</u>	<u>Jak3</u>	<u>STATS</u>	<u>GAS(elements) or ISRE</u>
<u>IFN family</u>						
IFN-a/B	+	+	-	-	1,2,3	ISRE
IFN-g		+	+	-	1	GAS (IRF1>Lys6>IFP)
Il-10	+	?	?	-	1,3	
<u>gp130 family</u>						
IL-6 (Pleiotropic)	+	+	+	?	1,3	GAS (IRF1>Lys6>IFP)
Il-11(Pleiotropic)	?	+	?	?	1,3	
OnM(Pleiotropic)	?	+	+	?	1,3	
LIF(Pleiotropic)	?	+	+	?	1,3	
CNTF(Pleiotropic)	-/+	+	+	?	1,3	
G-CSF(Pleiotropic)	?	+	?	?	1,3	
IL-12(Pleiotropic)	+	-	+	+	1,3	
<u>g-C family</u>						
IL-2 (lymphocytes)	-	+	-	+	1,3,5	GAS
IL-4 (lymph/myeloid)	-	+	-	+	6	GAS(IRF1=IFP
>>Ly6)(IgH)						
IL-7 (lymphocytes)	-	+	-	+	5	GAS
IL-9 (lymphocytes)	-	+	-	+	5	GAS
IL-13 (lymphocyte)	-	+	?	?	6	GAS
IL-15	?	+	?	+	5	GAS
<u>gp140 family</u>						
IL-3 (myeloid)	-	-	+	-	5	GAS (IRF1>IFP>>Ly6)
IL-5 (myeloid)	-	-	+	-	5	GAS
GM-CSF (myeloid)	-	-	+	-	5	GAS
<u>Growth hormone family</u>						
GH	?	-	+	-	5	
PRL	?	+/-	+	-	1,3,5	
EPO	?	-	+	-	5	GAS(B-CAS>IRF1=IFP>>Ly6)
<u>Receptor Tyrosine Kinases</u>						
EGF	?	+	+	-	1,3	GAS (IRF1)
PDGF	?	+	+	-	1,3	
CSF-1	?	+	+	-	1,3	GAS (not IRF1)

To construct a synthetic GAS containing promoter element, which is used in the Biological Assays described in Examples 32-33, a PCR based strategy is employed to generate a GAS-SV40 promoter sequence. The 5' primer contains four tandem copies of the GAS binding site found in the IRF1 promoter and previously demonstrated to bind STATs upon induction with a range of cytokines (Rothman et al., Immunity 1:457-468 (1994).), although other GAS or ISRE elements can be used instead. The 5' primer also contains 18bp of sequence complementary to the SV40 early promoter sequence and is flanked with an XhoI site. The sequence of the 5' primer is:

5':GCGCCTCGAGATTTCCTCCCGAAATCTAGATTTCCTCCCGAAATGATTTCCTCCCGAAATGATTTCCTCCCGAAATATCTGCCATCTCAATTAG:3' (SEQ ID NO: 38)

The downstream primer is complementary to the SV40 promoter and is flanked with a Hind III site: 5':GCGGCAAGCTTTTGTCAAAGCCTAGGC:3' (SEQ ID NO: 39)

PCR amplification is performed using the SV40 promoter template present in the B-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI/Hind III and subcloned into BLSK2-. (Stratagene.) Sequencing with forward and reverse primers confirms that the insert contains the following sequence:

5':CTCGAGATTTCCTCCCGAAATCTAGATTTCCTCCCGAAATGATTTCCTCCCGAAATGATTTCCTCCCGAAATATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCCATCCCGCCCCCTAACTCCGCCCCAGTTCCGCCCCATTCTCCGCCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCCTAGGCTTTTGCAAAAAGCTT:3' (SEQ ID NO: 40)

With this GAS promoter element linked to the SV40 promoter, a GAS:SEAP2 reporter construct is next engineered. Here, the reporter molecule is a secreted alkaline phosphatase, or "SEAP." Clearly, however, any reporter molecule can be instead of SEAP, in this or in any of the other Examples. Well known reporter molecules that can be used instead of SEAP include chloramphenicol acetyltransferase (CAT), luciferase, alkaline phosphatase, B-galactosidase, green fluorescent protein (GFP), or any protein detectable by an antibody.

The above sequence confirmed synthetic GAS-SV40 promoter element is subcloned into the pSEAP-Promoter vector obtained from Clontech using HindIII and XhoI, effectively replacing the SV40 promoter with the amplified GAS:SV40 promoter element, to create the GAS-SEAP vector. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

Thus, in order to generate mammalian stable cell lines expressing the GAS-SEAP reporter, the GAS-SEAP cassette is removed from the GAS-SEAP vector using Sall and

NotI, and inserted into a backbone vector containing the neomycin resistance gene, such as pGFP-1 (Clontech), using these restriction sites in the multiple cloning site, to create the GAS-SEAP/Neo vector. Once this vector is transfected into mammalian cells, this vector can then be used as a reporter molecule for GAS binding as described in Examples 32-33.

5 Other constructs can be made using the above description and replacing GAS with a different promoter sequence. For example, construction of reporter molecules containing EGR and NF-KB promoter sequences are described in Examples 34 and 35. However, many other promoters can be substituted using the protocols described in these Examples. For instance, SRE, IL-2, NFAT, or Osteocalcin promoters can be substituted, alone or in
10 combination (e.g., GAS/NF-KB/EGR, GAS/NF-KB, IL-2/NFAT, or NF-KB/GAS). Similarly, other cell lines can be used to test reporter construct activity, such as HELA (epithelial), HUVEC (endothelial), Reh (B-cell), Saos-2 (osteoblast), HUVAC (aortic), or Cardiomyocyte.

15 *Example 30: Assay for SEAP Activity*

As a reporter molecule for the assays described in examples disclosed herein, SEAP activity is assayed using the Tropix Phospho-light Kit (Cat. BP-400) according to the following general procedure. The Tropix Phospho-light Kit supplies the Dilution, Assay, and Reaction Buffers used below.

20 Prime a dispenser with the 2.5x Dilution Buffer and dispense 15 ul of 2.5x dilution buffer into Optiplates containing 35 ul of a solution containing an albumin fusion protein of the invention. Seal the plates with a plastic sealer and incubate at 65 degree C for 30 min. Separate the Optiplates to avoid uneven heating.

Cool the samples to room temperature for 15 minutes. Empty the dispenser and prime
25 with the Assay Buffer. Add 50 ml Assay Buffer and incubate at room temperature 5 min. Empty the dispenser and prime with the Reaction Buffer (see the Table below). Add 50 ul Reaction Buffer and incubate at room temperature for 20 minutes. Since the intensity of the chemiluminescent signal is time dependent, and it takes about 10 minutes to read 5 plates on a luminometer, thus one should treat 5 plates at each time and start the second set 10 minutes
30 later.

Read the relative light unit in the luminometer. Set H12 as blank, and print the results. An increase in chemiluminescence indicates reporter activity.

Reaction Buffer Formulation:

# of plates	Rxn buffer diluent (ml)	CSPD (ml)
10	60	3
11	65	3.25

12	70	3.5
13	75	3.75
14	80	4
15	85	4.25
16	90	4.5
17	95	4.75
18	100	5
19	105	5.25
20	110	5.5
21	115	5.75
22	120	6
23	125	6.25
24	130	6.5
25	135	6.75
26	140	7
27	145	7.25
28	150	7.5
29	155	7.75
30	160	8
31	165	8.25
32	170	8.5
33	175	8.75
34	180	9
35	185	9.25
36	190	9.5
37	195	9.75
38	200	10
39	205	10.25
40	210	10.5
41	215	10.75
42	220	11
43	225	11.25
44	230	11.5
45	235	11.75
46	240	12
47	245	12.25
48	250	12.5
49	255	12.75
50	260	13

Example 31: Assay Identifying Neuronal Activity.

When cells undergo differentiation and proliferation, a group of genes are activated through many different signal transduction pathways. One of these genes, EGR1 (early growth response gene 1), is induced in various tissues and cell types upon activation. The promoter of EGR1 is responsible for such induction. Using the EGR1 promoter linked to reporter molecules, the ability of fusion proteins of the invention to activate cells can be assessed.

Particularly, the following protocol is used to assess neuronal activity in PC12 cell

lines. PC12 cells (rat phenochromocytoma cells) are known to proliferate and/or differentiate by activation with a number of mitogens, such as TPA (tetradecanoyl phorbol acetate), NGF (nerve growth factor), and EGF (epidermal growth factor). The EGR1 gene expression is activated during this treatment. Thus, by stably transfecting PC12 cells with a construct
5 containing an EGR promoter linked to SEAP reporter, activation of PC12 cells by an albumin fusion protein of the present invention can be assessed.

The EGR/SEAP reporter construct can be assembled by the following protocol. The EGR-1 promoter sequence (-633 to +1)(Sakamoto K et al., Oncogene 6:867-871 (1991)) can be PCR amplified from human genomic DNA using the following primers:

10 5' GCGCTCGAGGGATGACAGCGATAGAACCCCGG-3' (SEQ ID NO: 41)
5' GCGAAGCTTCGCGACTCCCCGGATCCGCCTC-3' (SEQ ID NO: 42)

Using the GAS:SEAP/Neo vector produced in Example 29, EGR1 amplified product can then be inserted into this vector. Linearize the GAS:SEAP/Neo vector using restriction enzymes XhoI/HindIII, removing the GAS/SV40 stuffer. Restrict the EGR1 amplified
15 product with these same enzymes. Ligate the vector and the EGR1 promoter.

To prepare 96 well-plates for cell culture, two mls of a coating solution (1:30 dilution of collagen type I (Upstate Biotech Inc. Cat#08-115) in 30% ethanol (filter sterilized)) is added per one 10 cm plate or 50 ml per well of the 96-well plate, and allowed to air dry for 2 hr.

20 PC12 cells are routinely grown in RPMI-1640 medium (Bio Whittaker) containing 10% horse serum (JRH BIOSCIENCES, Cat. # 12449-78P), 5% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 ug/ml streptomycin on a precoated 10 cm tissue culture dish. One to four split is done every three to four days. Cells are removed from the plates by scraping and resuspended with pipetting up and down
25 for more than 15 times.

Transfect the EGR/SEAP/Neo construct into PC12 using techniques known in the art. EGR-SEAP/PC12 stable cells are obtained by growing the cells in 300 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 300 ug/ml G418 for couple of passages.

30 To assay for neuronal activity, a 10 cm plate with cells around 70 to 80% confluent is screened by removing the old medium. Wash the cells once with PBS (Phosphate buffered saline). Then starve the cells in low serum medium (RPMI-1640 containing 1% horse serum and 0.5% FBS with antibiotics) overnight.

The next morning, remove the medium and wash the cells with PBS. Scrape off the
35 cells from the plate, suspend the cells well in 2 ml low serum medium. Count the cell number and add more low serum medium to reach final cell density as 5×10^5 cells/ml.

Add 200 ul of the cell suspension to each well of 96-well plate (equivalent to 1×10^5 cells/well). Add a series of different concentrations of an albumin fusion protein of the invention, 37 degree C for 48 to 72 hr. As a positive control, a growth factor known to activate PC12 cells through EGR can be used, such as 50 ng/ul of Neuronal Growth Factor (NGF). Over fifty-fold induction of SEAP is typically seen in the positive control wells. SEAP assay may be routinely performed using techniques known in the art and/or as described in Example 30.

Example 32: Assay for T-cell Activity.

The following protocol is used to assess T-cell activity by identifying factors, and determining whether an albumin fusion protein of the invention proliferates and/or differentiates T-cells. T-cell activity is assessed using the GAS/SEAP/Neo construct produced in Example 29. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The T-cell used in this assay is Jurkat T-cells (ATCC Accession No. TIB-152), although Molt-3 cells (ATCC Accession No. CRL-1552) and Molt-4 cells (ATCC Accession No. CRL-1582) cells can also be used.

Jurkat T-cells are lymphoblastic CD4+ Th1 helper cells. In order to generate stable cell lines, approximately 2 million Jurkat cells are transfected with the GAS-SEAP/neo vector using DMRIE-C (Life Technologies)(transfection procedure described below). The transfected cells are seeded to a density of approximately 20,000 cells per well and transfectants resistant to 1 mg/ml gentamicin selected. Resistant colonies are expanded and then tested for their response to increasing concentrations of interferon gamma. The dose response of a selected clone is demonstrated.

Specifically, the following protocol will yield sufficient cells for 75 wells containing 200 ul of cells. Thus, it is either scaled up, or performed in multiple to generate sufficient cells for multiple 96 well plates. Jurkat cells are maintained in RPMI + 10% serum with 1% Pen-Strep. Combine 2.5 mls of OPTI-MEM (Life Technologies) with 10 ug of plasmid DNA in a T25 flask. Add 2.5 ml OPTI-MEM containing 50 ul of DMRIE-C and incubate at room temperature for 15-45 mins.

During the incubation period, count cell concentration, spin down the required number of cells (10^7 per transfection), and resuspend in OPTI-MEM to a final concentration of 10^7 cells/ml. Then add 1ml of 1×10^7 cells in OPTI-MEM to T25 flask and incubate at 37 degree C for 6 hrs. After the incubation, add 10 ml of RPMI + 15% serum.

The Jurkat:GAS-SEAP stable reporter lines are maintained in RPMI + 10% serum, 1 mg/ml Gentamicin, and 1% Pen-Strep. These cells are treated with varying concentrations of one or more fusion proteins of the present invention.

On the day of treatment with the fusion protein, the cells should be washed and resuspended in fresh RPMI + 10% serum to a density of 500,000 cells per ml. The exact number of cells required will depend on the number of fusion proteins and the number of different concentrations of fusion proteins being screened. For one 96 well plate,
5 approximately 10 million cells (for 10 plates, 100 million cells) are required.

The well dishes containing Jurkat cells treated with the fusion protein are placed in an incubator for 48 hrs (note: this time is variable between 48-72 hrs). 35 ul samples from each well are then transferred to an opaque 96 well plate using a 12 channel pipette. The opaque plates should be covered (using sellophane covers) and stored at -20 degree C until SEAP
10 assays are performed according to Example 30. The plates containing the remaining treated cells are placed at 4 degree C and serve as a source of material for repeating the assay on a specific well if desired.

As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate Jurkat T cells. Over 30 fold induction is typically observed in the positive control
15 wells.

The above protocol may be used in the generation of both transient, as well as, stable transfected cells, which would be apparent to those of skill in the art.

Example 33: Assay for T-cell Activity

20 NF-KB (Nuclear Factor KB) is a transcription factor activated by a wide variety of agents including the inflammatory cytokines IL-1 and TNF, CD30 and CD40, lymphotoxin-alpha and lymphotoxin-beta, by exposure to LPS or thrombin, and by expression of certain viral gene products. As a transcription factor, NF-KB regulates the expression of genes involved in immune cell activation, control of apoptosis (NF-KB appears to shield cells from
25 apoptosis), B and T-cell development, anti-viral and antimicrobial responses, and multiple stress responses.

In non-stimulated conditions, NF-KB is retained in the cytoplasm with I-KB (Inhibitor KB). However, upon stimulation, I-KB is phosphorylated and degraded, causing NF-KB to shuttle to the nucleus, thereby activating transcription of target genes. Target
30 genes activated by NF-KB include IL-2, IL-6, GM-CSF, ICAM-1 and class 1 MHC.

Due to its central role and ability to respond to a range of stimuli, reporter constructs utilizing the NF-KB promoter element are used to screen the fusion protein. Activators or inhibitors of NF-KB would be useful in treating, preventing, and/or diagnosing diseases. For example, inhibitors of NF-KB could be used to treat those diseases related to the acute or
35 chronic activation of NF-KB, such as rheumatoid arthritis.

To construct a vector containing the NF-KB promoter element, a PCR based strategy

is employed. The upstream primer contains four tandem copies of the NF-KB binding site (GGGGACTTTCCC) (SEQ ID NO: 43), 18 bp of sequence complementary to the 5' end of the SV40 early promoter sequence, and is flanked with an XhoI site:

5':GCGGCCTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGA
5 CTTTCCATCCTGCCATCTCAATTAG:3' (SEQ ID NO: 44)

The downstream primer is complementary to the 3' end of the SV40 promoter and is flanked with a Hind III site:

5':GCGGCAAGCTTTTGTCAAAGCCTAGGC:3' (SEQ ID NO: 39)

10 PCR amplification is performed using the SV40 promoter template present in the pB-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI and Hind III and subcloned into BLSK2-. (Stratagene) Sequencing with the T7 and T3 primers confirms the insert contains the following sequence:

5':CTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGGACTTTCC
ATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCCATCCC
15 GCCCCCTAACTCCGCCCCAGTTCCGCCCCATTCTCCGCCCCATGGCTGACTAATTTTTT
TTATTTATGCAGAGGCCGAGGCCGCTCGGCCTCTGAGCTATTCCAGAAGTAGTG
AGGAGGCTTTTTTGGAGGCCTAGGCTTTTGC AAAAAGCTT:3' (SEQ ID NO: 45)

20 Next, replace the SV40 minimal promoter element present in the pSEAP2-promoter plasmid (Clontech) with this NF-KB/SV40 fragment using XhoI and HindIII. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

25 In order to generate stable mammalian cell lines, the NF-KB/SV40/SEAP cassette is removed from the above NF-KB/SEAP vector using restriction enzymes SalI and NotI, and inserted into a vector containing neomycin resistance. Particularly, the NF-KB/SV40/SEAP cassette was inserted into pGFP-1 (Clontech), replacing the GFP gene, after restricting pGFP-1 with SalI and NotI.

30 Once NF-KB/SV40/SEAP/Neo vector is created, stable Jurkat T-cells are created and maintained according to the protocol described in Example 32. Similarly, the method for assaying fusion proteins with these stable Jurkat T-cells is also described in Example 32. As a positive control, exogenous TNF alpha (0.1, 1, 10 ng) is added to wells H9, H10, and H11, with a 5-10 fold activation typically observed.

Example 33: Assay Identifying Myeloid Activity

35 The following protocol is used to assess myeloid activity of an albumin fusion protein of the present invention by determining whether the fusion protein proliferates and/or differentiates myeloid cells. Myeloid cell activity is assessed using the GAS/SEAP/Neo

construct produced in Example 29. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The myeloid cell used in this assay is U937, a pre-monocyte cell line, although TF-1, HL60, or KG1 can be used.

To transiently transfect U937 cells with the GAS/SEAP/Neo construct produced in Example 29, a DEAE-Dextran method (Kharbanda et. al., 1994, Cell Growth & Differentiation, 5:259-265) is used. First, harvest 2×10^7 U937 cells and wash with PBS. The U937 cells are usually grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

Next, suspend the cells in 1 ml of 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 mg/ml DEAE-Dextran, 8 ug GAS-SEAP2 plasmid DNA, 140 mM NaCl, 5 mM KCl, 375 uM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1 mM MgCl_2 , and 675 uM CaCl_2 . Incubate at 37 degrees C for 45 min.

Wash the cells with RPMI 1640 medium containing 10% FBS and then resuspend in 10 ml complete medium and incubate at 37 degree C for 36 hr.

The GAS-SEAP/U937 stable cells are obtained by growing the cells in 400 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 400 ug/ml G418 for couple of passages.

These cells are tested by harvesting 1×10^8 cells (this is enough for ten 96-well plates assay) and wash with PBS. Suspend the cells in 200 ml above described growth medium, with a final density of 5×10^5 cells/ml. Plate 200 ul cells per well in the 96-well plate (or 1×10^5 cells/well).

Add different concentrations of the fusion protein. Incubate at 37 degree C for 48 to 72 hr. As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate U937 cells. Over 30 fold induction is typically observed in the positive control wells.

SEAP assay the supernatant according to methods known in the art and/or the protocol described in Example 30.

Example 34: Assay Identifying Changes in Small Molecule Concentration and Membrane Permeability

Binding of a ligand to a receptor is known to alter intracellular levels of small molecules, such as calcium, potassium, sodium, and pH, as well as alter membrane potential. These alterations can be measured in an assay to identify fusion proteins which bind to receptors of a particular cell. Although the following protocol describes an assay for calcium, this protocol can easily be modified to detect changes in potassium, sodium, pH, membrane potential, or any other small molecule which is detectable by a fluorescent probe.

The following assay uses Fluorometric Imaging Plate Reader ("FLIPR") to measure

changes in fluorescent molecules (Molecular Probes) that bind small molecules. Clearly, any fluorescent molecule detecting a small molecule can be used instead of the calcium fluorescent molecule, fluo-4 (Molecular Probes, Inc.; catalog no. F-14202), used here.

For adherent cells, seed the cells at 10,000 -20,000 cells/well in a Co-star black 96-well plate with clear bottom. The plate is incubated in a CO₂ incubator for 20 hours. The adherent cells are washed two times in Biotek washer with 200 ul of HBSS (Hank's Balanced Salt Solution) leaving 100 ul of buffer after the final wash.

A stock solution of 1 mg/ml fluo-4 is made in 10% pluronic acid DMSO. To load the cells with fluo-4, 50 ul of 12 ug/ml fluo-4 is added to each well. The plate is incubated at 37 degrees C in a CO₂ incubator for 60 min. The plate is washed four times in the Biotek washer with HBSS leaving 100 ul of buffer.

For non-adherent cells, the cells are spun down from culture media. Cells are re-suspended to 2-5x10⁶ cells/ml with HBSS in a 50-ml conical tube. 4 ul of 1 mg/ml fluo-4 solution in 10% pluronic acid DMSO is added to each ml of cell suspension. The tube is then placed in a 37 degrees C water bath for 30-60 min. The cells are washed twice with HBSS, resuspended to 1x10⁶ cells/ml, and dispensed into a microplate, 100 ul/well. The plate is centrifuged at 1000 rpm for 5 min. The plate is then washed once in Denley Cell Wash with 200 ul, followed by an aspiration step to 100 ul final volume.

For a non-cell based assay, each well contains a fluorescent molecule, such as fluo-4. The fusion protein of the invention is added to the well, and a change in fluorescence is detected.

To measure the fluorescence of intracellular calcium, the FLIPR is set for the following parameters: (1) System gain is 300-800 mW; (2) Exposure time is 0.4 second; (3) Camera F/stop is F/2; (4) Excitation is 488 nm; (5) Emission is 530 nm; and (6) Sample addition is 50 ul. Increased emission at 530 nm indicates an extracellular signaling event caused by an albumin fusion protein of the present invention or a molecule induced by an albumin fusion protein of the present invention, which has resulted in an increase in the intracellular Ca⁺⁺ concentration.

Example 35: Assay Identifying Tyrosine Kinase Activity

The Protein Tyrosine Kinases (PTK) represent a diverse group of transmembrane and cytoplasmic kinases. Within the Receptor Protein Tyrosine Kinase (RPTK) group are receptors for a range of mitogenic and metabolic growth factors including the PDGF, FGF, EGF, NGF, HGF and Insulin receptor subfamilies. In addition there are a large family of RPTKs for which the corresponding ligand is unknown. Ligands for RPTKs include mainly secreted small proteins, but also membrane-bound and extracellular matrix proteins.

Activation of RPTK by ligands involves ligand-mediated receptor dimerization, resulting in transphosphorylation of the receptor subunits and activation of the cytoplasmic tyrosine kinases. The cytoplasmic tyrosine kinases include receptor associated tyrosine kinases of the src-family (e.g., src, yes, lck, lyn, fyn) and non-receptor linked and cytosolic protein tyrosine kinases, such as the Jak family, members of which mediate signal transduction triggered by the cytokine superfamily of receptors (e.g., the Interleukins, Interferons, GM-CSF, and Leptin).

Because of the wide range of known factors capable of stimulating tyrosine kinase activity, identifying whether an albumin fusion protein of the present invention or a molecule induced by a fusion protein of the present invention is capable of activating tyrosine kinase signal transduction pathways is of interest. Therefore, the following protocol is designed to identify such molecules capable of activating the tyrosine kinase signal transduction pathways.

Seed target cells (e.g., primary keratinocytes) at a density of approximately 25,000 cells per well in a 96 well Loprodyne Silent Screen Plates purchased from Nalge Nunc (Naperville, IL). The plates are sterilized with two 30 minute rinses with 100% ethanol, rinsed with water and dried overnight. Some plates are coated for 2 hr with 100 ml of cell culture grade type I collagen (50 mg/ml), gelatin (2%) or polylysine (50 mg/ml), all of which can be purchased from Sigma Chemicals (St. Louis, MO) or 10% Matrigel purchased from Becton Dickinson (Bedford, MA), or calf serum, rinsed with PBS and stored at 4 degree C. Cell growth on these plates is assayed by seeding 5,000 cells/well in growth medium and indirect quantitation of cell number through use of alamarBlue as described by the manufacturer Alamar Biosciences, Inc. (Sacramento, CA) after 48 hr. Falcon plate covers #3071 from Becton Dickinson (Bedford, MA) are used to cover the Loprodyne Silent Screen Plates. Falcon Microtest III cell culture plates can also be used in some proliferation experiments.

To prepare extracts, A431 cells are seeded onto the nylon membranes of Loprodyne plates (20,000/200ml/well) and cultured overnight in complete medium. Cells are quiesced by incubation in serum-free basal medium for 24 hr. After 5-20 minutes treatment with EGF (60ng/ml) or a different concentrations of an albumin fusion protein of the invention, the medium was removed and 100 ml of extraction buffer ((20 mM HEPES pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 2 mM Na₃VO₄, 2 mM Na₄P₂O₇ and a cocktail of protease inhibitors (# 1836170) obtained from Boehringer Mannheim (Indianapolis, IN)) is added to each well and the plate is shaken on a rotating shaker for 5 minutes at 4°C. The plate is then placed in a vacuum transfer manifold and the extract filtered through the 0.45 mm membrane bottoms of each well using house vacuum. Extracts are collected in a 96-well

catch/assay plate in the bottom of the vacuum manifold and immediately placed on ice. To obtain extracts clarified by centrifugation, the content of each well, after detergent solubilization for 5 minutes, is removed and centrifuged for 15 minutes at 4 degree C at 16,000 x g.

5 Test the filtered extracts for levels of tyrosine kinase activity. Although many methods of detecting tyrosine kinase activity are known, one method is described here.

 Generally, the tyrosine kinase activity of an albumin fusion protein of the invention is evaluated by determining its ability to phosphorylate a tyrosine residue on a specific substrate (a biotinylated peptide). Biotinylated peptides that can be used for this purpose include PSK1
10 (corresponding to amino acids 6-20 of the cell division kinase cdc2-p34) and PSK2 (corresponding to amino acids 1-17 of gastrin). Both peptides are substrates for a range of tyrosine kinases and are available from Boehringer Mannheim.

 The tyrosine kinase reaction is set up by adding the following components in order. First, add 10ul of 5uM Biotinylated Peptide, then 10ul ATP/Mg₂₊ (5mM ATP/50mM
15 MgCl₂), then 10ul of 5x Assay Buffer (40mM imidazole hydrochloride, pH7.3, 40 mM beta-glycerophosphate, 1mM EGTA, 100mM MgCl₂, 5 mM MnCl₂, 0.5 mg/ml BSA), then 5ul of Sodium Vanadate(1mM), and then 5ul of water. Mix the components gently and preincubate the reaction mix at 30 degree C for 2 min. Initiate the reaction by adding 10ul of the control enzyme or the filtered supernatant.

20 The tyrosine kinase assay reaction is then terminated by adding 10 ul of 120mM EDTA and place the reactions on ice.

 Tyrosine kinase activity is determined by transferring 50 ul aliquot of reaction mixture to a microtiter plate (MTP) module and incubating at 37 degree C for 20 min. This allows the streptavidin coated 96 well plate to associate with the biotinylated peptide. Wash the MTP
25 module with 300ul/well of PBS four times. Next add 75 ul of anti-phosphotyrosine antibody conjugated to horse radish peroxidase(anti-P-Tyr-POD(0.5u/ml)) to each well and incubate at 37 degree C for one hour. Wash the well as above.

 Next add 100ul of peroxidase substrate solution (Boehringer Mannheim) and incubate at room temperature for at least 5 mins (up to 30 min). Measure the absorbance of the sample
30 at 405 nm by using ELISA reader. The level of bound peroxidase activity is quantitated using an ELISA reader and reflects the level of tyrosine kinase activity.

Example 36: Assay Identifying Phosphorylation Activity

 As a potential alternative and/or complement to the assay of protein tyrosine kinase
35 activity described in Example 35, an assay which detects activation (phosphorylation) of major intracellular signal transduction intermediates can also be used. For example, as

described below one particular assay can detect tyrosine phosphorylation of the Erk-1 and Erk-2 kinases. However, phosphorylation of other molecules, such as Raf, JNK, p38 MAP, Map kinase kinase (MEK), MEK kinase, Src, Muscle specific kinase (MuSK), IRAK, Tec, and Janus, as well as any other phosphoserine, phosphotyrosine, or phosphothreonine molecule, can be detected by substituting these molecules for Erk-1 or Erk-2 in the following assay.

Specifically, assay plates are made by coating the wells of a 96-well ELISA plate with 0.1ml of protein G (1ug/ml) for 2 hr at room temp, (RT). The plates are then rinsed with PBS and blocked with 3% BSA/PBS for 1 hr at RT. The protein G plates are then treated with 2 commercial monoclonal antibodies (100ng/well) against Erk-1 and Erk-2 (1 hr at RT) (Santa Cruz Biotechnology). (To detect other molecules, this step can easily be modified by substituting a monoclonal antibody detecting any of the above described molecules.) After 3-5 rinses with PBS, the plates are stored at 4 degree C until use.

A431 cells are seeded at 20,000/well in a 96-well Loprodyne filterplate and cultured overnight in growth medium. The cells are then starved for 48 hr in basal medium (DMEM) and then treated with EGF (6ng/well) or varying concentrations of the fusion protein of the invention for 5-20 minutes. The cells are then solubilized and extracts filtered directly into the assay plate.

After incubation with the extract for 1 hr at RT, the wells are again rinsed. As a positive control, a commercial preparation of MAP kinase (10ng/well) is used in place of A431 extract. Plates are then treated with a commercial polyclonal (rabbit) antibody (1ug/ml) which specifically recognizes the phosphorylated epitope of the Erk-1 and Erk-2 kinases (1 hr at RT). This antibody is biotinylated by standard procedures. The bound polyclonal antibody is then quantitated by successive incubations with Europium-streptavidin and Europium fluorescence enhancing reagent in the Wallac DELFIA instrument (time-resolved fluorescence). An increased fluorescent signal over background indicates a phosphorylation by the fusion protein of the present invention or a molecule induced by an albumin fusion protein of the present invention.

Example 37: Assay for the Stimulation of Bone Marrow CD34+ Cell Proliferation

This assay is based on the ability of human CD34+ to proliferate in the presence of hematopoietic growth factors and evaluates the ability of fusion proteins of the invention to stimulate proliferation of CD34+ cells.

It has been previously shown that most mature precursors will respond to only a single signal. More immature precursors require at least two signals to respond. Therefore,

to test the effect of fusion proteins of the invention on hematopoietic activity of a wide range of progenitor cells, the assay contains a given fusion protein of the invention in the presence or absence of hematopoietic growth factors. Isolated cells are cultured for 5 days in the presence of Stem Cell Factor (SCF) in combination with tested sample. SCF alone has a very limited effect on the proliferation of bone marrow (BM) cells, acting in such conditions only as a "survival" factor. However, combined with any factor exhibiting stimulatory effect on these cells (e.g., IL-3), SCF will cause a synergistic effect. Therefore, if the tested fusion protein has a stimulatory effect on hematopoietic progenitors, such activity can be easily detected. Since normal BM cells have a low level of cycling cells, it is likely that any inhibitory effect of a given fusion protein might not be detected. Accordingly, assays for an inhibitory effect on progenitors is preferably tested in cells that are first subjected to *in vitro* stimulation with SCF+IL-3, and then contacted with the compound that is being evaluated for inhibition of such induced proliferation.

Briefly, CD34+ cells are isolated using methods known in the art. The cells are thawed and resuspended in medium (QBSF 60 serum-free medium with 1% L-glutamine (500ml) Quality Biological, Inc., Gaithersburg, MD Cat# 160-204-101). After several gentle centrifugation steps at 200 x g, cells are allowed to rest for one hour. The cell count is adjusted to 2.5×10^5 cells/ml. During this time, 100 μ l of sterile water is added to the peripheral wells of a 96-well plate. The cytokines that can be tested with an albumin fusion protein of the invention in this assay is rhSCF (R&D Systems, Minneapolis, MN, Cat# 255-SC) at 50 ng/ml alone and in combination with rhSCF and rhIL-3 (R&D Systems, Minneapolis, MN, Cat# 203-ML) at 30 ng/ml. After one hour, 10 μ l of prepared cytokines, varying concentrations of an albumin fusion protein of the invention, and 20 μ l of diluted cells are added to the media which is already present in the wells to allow for a final total volume of 100 μ l. The plates are then placed in a 37°C/5% CO₂ incubator for five days.

Eighteen hours before the assay is harvested, 0.5 μ Ci/well of [3H] Thymidine is added in a 10 μ l volume to each well to determine the proliferation rate. The experiment is terminated by harvesting the cells from each 96-well plate to a filtermat using the Tomtec Harvester 96. After harvesting, the filtermats are dried, trimmed and placed into OmniFilter assemblies consisting of one OmniFilter plate and one OmniFilter Tray. 60 μ l Microscint is added to each well and the plate sealed with TopSeal-A press-on sealing film. A bar code sticker is affixed to the first plate for counting. The sealed plates are then loaded and the level of radioactivity determined via the Packard Top Count and the printed data collected for

analysis. The level of radioactivity reflects the amount of cell proliferation.

The studies described in this example test the activity of a given fusion protein to stimulate bone marrow CD34+ cell proliferation. One skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy) as well as agonists and antagonists thereof. The ability of an albumin fusion protein of the invention to stimulate the proliferation of bone marrow CD34+ cells indicates that the albumin fusion protein and/or polynucleotides corresponding to the fusion protein are useful for the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein.

Example 38: Assay for Extracellular Matrix Enhanced Cell Response (EMECCR)

The objective of the Extracellular Matrix Enhanced Cell Response (EMECCR) assay is to evaluate the ability of fusion proteins of the invention to act on hematopoietic stem cells in the context of the extracellular matrix (ECM) induced signal.

Cells respond to the regulatory factors in the context of signal(s) received from the surrounding microenvironment. For example, fibroblasts, and endothelial and epithelial stem cells fail to replicate in the absence of signals from the ECM. Hematopoietic stem cells can undergo self-renewal in the bone marrow, but not in *in vitro* suspension culture. The ability of stem cells to undergo self-renewal *in vitro* is dependent upon their interaction with the stromal cells and the ECM protein fibronectin (fn). Adhesion of cells to fn is mediated by the $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrin receptors, which are expressed by human and mouse hematopoietic stem cells. The factor(s) which integrate with the ECM environment and are responsible for stimulating stem cell self-renewal have not yet been identified. Discovery of such factors should be of great interest in gene therapy and bone marrow transplant applications.

Briefly, polystyrene, non tissue culture treated, 96-well plates are coated with fn fragment at a coating concentration of 0.2 $\mu\text{g}/\text{cm}^2$. Mouse bone marrow cells are plated (1,000 cells/well) in 0.2 ml of serum-free medium. Cells cultured in the presence of IL-3 (5 ng/ml) + SCF (50 ng/ml) would serve as the positive control, conditions under which little self-renewal but pronounced differentiation of the stem cells is to be expected. Albumin fusion proteins of the invention are tested with appropriate negative controls in the presence and absence of SCF(5.0 ng/ml), where volume of the administered composition containing the albumin fusion protein of the invention represents 10% of the total assay volume. The plated cells are then allowed to grow by incubating in a low oxygen environment (5% CO_2 , 7% O_2 ,

and 88% N₂) tissue culture incubator for 7 days. The number of proliferating cells within the wells is then quantitated by measuring thymidine incorporation into cellular DNA. Verification of the positive hits in the assay will require phenotypic characterization of the cells, which can be accomplished by scaling up of the culture system and using appropriate antibody reagents against cell surface antigens and FACScan.

One skilled in the art could easily modify the exemplified studies to test the activity of albumin fusion proteins and polynucleotides of the invention (e.g., gene therapy).

If a particular fusion protein of the present invention is found to be a stimulator of hematopoietic progenitors, the fusion protein and polynucleotides corresponding to the fusion protein may be useful for example, in the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein. The fusion protein may also be useful in the expansion of stem cells and committed progenitors of various blood lineages, and in the differentiation and/or proliferation of various cell types.

Additionally, the albumin fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention, may also be employed to inhibit the proliferation and differentiation of hematopoietic cells and therefore may be employed to protect bone marrow stem cells from chemotherapeutic agents during chemotherapy. This antiproliferative effect may allow administration of higher doses of chemotherapeutic agents and, therefore, more effective chemotherapeutic treatment.

Moreover, fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention may also be useful for the treatment and diagnosis of hematopoietic related disorders such as, anemia, pancytopenia, leukopenia, thrombocytopenia or leukemia, since stromal cells are important in the production of cells of hematopoietic lineages. The uses include bone marrow cell ex-vivo culture, bone marrow transplantation, bone marrow reconstitution, radiotherapy or chemotherapy of neoplasia.

Example 39: Human Dermal Fibroblast and Aortic Smooth Muscle Cell Proliferation

An albumin fusion protein of the invention is added to cultures of normal human dermal fibroblasts (NHDF) and human aortic smooth muscle cells (AoSMC) and two co-assays are performed with each sample. The first assay examines the effect of the fusion protein on the proliferation of normal human dermal fibroblasts (NHDF) or aortic smooth muscle cells (AoSMC). Aberrant growth of fibroblasts or smooth muscle cells is a part of several pathological processes, including fibrosis, and restenosis. The second assay examines IL6 production by both NHDF and SMC. IL6 production is an indication of

functional activation. Activated cells will have increased production of a number of cytokines and other factors, which can result in a proinflammatory or immunomodulatory outcome. Assays are run with and without co-TNF α stimulation, in order to check for costimulatory or inhibitory activity.

5 Briefly, on day 1, 96-well black plates are set up with 1000 cells/well (NHDF) or 2000 cells/well (AoSMC) in 100 μ l culture media. NHDF culture media contains: Clonetics FB basal media, 1mg/ml hFGF, 5mg/ml insulin, 50mg/ml gentamycin, 2%FBS, while AoSMC culture media contains Clonetics SM basal media, 0.5 μ g/ml hEGF, 5mg/ml insulin, 1 μ g/ml hFGF, 50mg/ml gentamycin, 50 μ g/ml Amphotericin B, 5%FBS. After incubation at
10 37°C for at least 4-5 hours culture media is aspirated and replaced with growth arrest media. Growth arrest media for NHDF contains fibroblast basal media, 50mg/ml gentamycin, 2% FBS, while growth arrest media for AoSMC contains SM basal media, 50mg/ml gentamycin, 50 μ g/ml Amphotericin B, 0.4% FBS. Incubate at 37 °C until day 2.

On day 2, serial dilutions and templates of an albumin fusion protein of the invention
15 are designed such that they always include media controls and known-protein controls. For both stimulation and inhibition experiments, proteins are diluted in growth arrest media. For inhibition experiments, TNF α is added to a final concentration of 2ng/ml (NHDF) or 5ng/ml (AoSMC). Add 1/3 vol media containing controls or an albumin fusion protein of the invention and incubate at 37 degrees C/5% CO $_2$ until day 5.

20 Transfer 60 μ l from each well to another labeled 96-well plate, cover with a plate-sealer, and store at 4 degrees C until Day 6 (for IL6 ELISA). To the remaining 100 μ l in the cell culture plate, aseptically add Alamar Blue in an amount equal to 10% of the culture volume (10 μ l). Return plates to incubator for 3 to 4 hours. Then measure fluorescence with
25 excitation at 530nm and emission at 590nm using the CytoFluor. This yields the growth stimulation/inhibition data.

On day 5, the IL6 ELISA is performed by coating a 96 well plate with 50-100 μ l/well of Anti-Human IL6 Monoclonal antibody diluted in PBS, pH 7.4, incubate ON at room temperature.

On day 6, empty the plates into the sink and blot on paper towels. Prepare Assay
30 Buffer containing PBS with 4% BSA. Block the plates with 200 μ l/well of Pierce Super Block blocking buffer in PBS for 1-2 hr and then wash plates with wash buffer (PBS, 0.05% Tween-20). Blot plates on paper towels. Then add 50 μ l/well of diluted Anti-Human IL-6 Monoclonal, Biotin-labeled antibody at 0.50 mg/ml. Make dilutions of IL-6 stock in media (30, 10, 3, 1, 0.3, 0 ng/ml). Add duplicate samples to top row of plate. Cover the plates and

incubate for 2 hours at RT on shaker.

Plates are washed with wash buffer and blotted on paper towels. Dilute EU-labeled Streptavidin 1:1000 in Assay buffer, and add 100 μ l/well. Cover the plate and incubate 1 h at RT. Plates are again washed with wash buffer and blotted on paper towels.

5 Add 100 μ l/well of Enhancement Solution. Shake for 5 minutes. Read the plate on the Wallac DELFIA Fluorometer. Readings from triplicate samples in each assay were tabulated and averaged.

A positive result in this assay suggests AoSMC cell proliferation and that the albumin fusion protein may be involved in dermal fibroblast proliferation and/or smooth muscle cell proliferation. A positive result also suggests many potential uses of the fusion protein and polynucleotides encoding the albumin fusion protein. For example, inflammation and immune responses, wound healing, and angiogenesis, as detailed throughout this specification. Particularly, fusion proteins may be used in wound healing and dermal regeneration, as well as the promotion of vasculogenesis, both of the blood vessels and lymphatics. The growth of vessels can be used in the treatment of, for example, cardiovascular diseases. Additionally, fusion proteins showing antagonistic activity in this assay may be useful in treating diseases, disorders, and/or conditions which involve angiogenesis by acting as an anti-vascular agent (e.g., anti-angiogenesis). These diseases, disorders, and/or conditions are known in the art and/or are described herein, such as, for example, malignancies, solid tumors, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; arteriosclerotic plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uveitis and Pterygia (abnormal blood vessel growth) of the eye; 25 rheumatoid arthritis; psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; fibromuscular dysplasia; 30 wound granulation; Crohn's disease; and atherosclerosis. Moreover, albumin fusion proteins that act as antagonists in this assay may be useful in treating anti-hyperproliferative diseases and/or anti-inflammatory known in the art and/or described herein.

35 *Example 40: Cellular Adhesion Molecule (CAM) Expression on Endothelial Cells*

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves

specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

Briefly, endothelial cells (e.g., Human Umbilical Vein Endothelial cells (HUVECs)) are grown in a standard 96 well plate to confluence, growth medium is removed from the cells and replaced with 100 μ l of 199 Medium (10% fetal bovine serum (FBS)). Samples for testing (containing an albumin fusion protein of the invention) and positive or negative controls are added to the plate in triplicate (in 10 μ l volumes). Plates are then incubated at 37°C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μ l of 0.1% paraformaldehyde-PBS(with Ca++ and Mg++) is added to each well. Plates are held at 4°C for 30 min. Fixative is removed from the wells and wells are washed 1X with PBS(+Ca,Mg) + 0.5% BSA and drained. 10 μ l of diluted primary antibody is added to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μ g/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed three times with PBS(+Ca,Mg) + 0.5% BSA. 20 μ l of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution, referred to herein as the working dilution) are added to each well and incubated at 37°C for 30 min. Wells are washed three times with PBS(+Ca,Mg)+0.5% BSA. Dissolve 1 tablet of p-Nitrophenol Phosphate pNPP per 5 ml of glycine buffer (pH 10.4). 100 μ l of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphatase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μ l of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μ l of pNPP reagent is then added to each of the standard wells. The plate is incubated at 37°C for 4h. A volume of 50 μ l of 3M NaOH is added to all wells. The plate is read on a plate reader at 405 nm using the background subtraction option on blank wells filled with glycine buffer only. Additionally, the template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

Example 41: Alamar Blue Endothelial Cells Proliferation Assay

This assay may be used to quantitatively determine protein mediated inhibition of bFGF-induced proliferation of Bovine Lymphatic Endothelial Cells (LECs), Bovine Aortic Endothelial Cells (BAECs) or Human Microvascular Uterine Myometrial Cells (UTMECs).

5 This assay incorporates a fluorometric growth indicator based on detection of metabolic activity. A standard Alamar Blue Proliferation Assay is prepared in EGM-2MV with 10 ng/ml of bFGF added as a source of endothelial cell stimulation. This assay may be used with a variety of endothelial cells with slight changes in growth medium and cell concentration. Dilutions of protein batches to be tested are diluted as appropriate. Serum-free medium
10 (GIBCO SFM) without bFGF is used as a non-stimulated control and Angiostatin or TSP-1 are included as a known inhibitory controls.

Briefly, LEC, BAECs or UTMECs are seeded in growth media at a density of 5000 to 2000 cells/well in a 96 well plate and placed at 37 degreesC overnight. After the overnight incubation of the cells, the growth media is removed and replaced with GIBCO EC-SFM.

15 The cells are treated with the appropriate dilutions of an albumin fusion protein of the invention or control protein sample(s) (prepared in SFM) in triplicate wells with additional bFGF to a concentration of 10 ng/ ml. Once the cells have been treated with the samples, the plate(s) is/are placed back in the 37° C incubator for three days. After three days 10 ml of stock alamar blue (Biosource Cat# DAL1100) is added to each well and the plate(s) is/are
20 placed back in the 37°C incubator for four hours. The plate(s) are then read at 530nm excitation and 590nm emission using the CytoFluor fluorescence reader. Direct output is recorded in relative fluorescence units.

Alamar blue is an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. As cells
25 grow in culture, innate metabolic activity results in a chemical reduction of the immediate surrounding environment. Reduction related to growth causes the indicator to change from oxidized (non-fluorescent blue) form to reduced (fluorescent red) form (i.e., stimulated proliferation will produce a stronger signal and inhibited proliferation will produce a weaker signal and the total signal is proportional to the total number of cells as well as their metabolic
30 activity). The background level of activity is observed with the starvation medium alone. This is compared to the output observed from the positive control samples (bFGF in growth medium) and protein dilutions.

Example 42: Detection of Inhibition of a Mixed Lymphocyte Reaction

35 This assay can be used to detect and evaluate inhibition of a Mixed Lymphocyte Reaction (MLR) by fusion proteins of the invention. Inhibition of a MLR may be due to a

direct effect on cell proliferation and viability, modulation of costimulatory molecules on interacting cells, modulation of adhesiveness between lymphocytes and accessory cells, or modulation of cytokine production by accessory cells. Multiple cells may be targeted by the albumin fusion proteins that inhibit MLR since the peripheral blood mononuclear fraction
5 used in this assay includes T, B and natural killer lymphocytes, as well as monocytes and dendritic cells.

Albumin fusion proteins of the invention found to inhibit the MLR may find application in diseases associated with lymphocyte and monocyte activation or proliferation. These include, but are not limited to, diseases such as asthma, arthritis, diabetes,
10 inflammatory skin conditions, psoriasis, eczema, systemic lupus erythematosus, multiple sclerosis, glomerulonephritis, inflammatory bowel disease, crohn's disease, ulcerative colitis, arteriosclerosis, cirrhosis, graft vs. host disease, host vs. graft disease, hepatitis, leukemia and lymphoma.

Briefly, PBMCs from human donors are purified by density gradient centrifugation
15 using Lymphocyte Separation Medium (LSM[®], density 1.0770 g/ml, Organon Teknika Corporation, West Chester, PA). PBMCs from two donors are adjusted to 2×10^6 cells/ml in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS and 2 mM glutamine. PBMCs from a third donor is adjusted to 2×10^5 cells/ml. Fifty microliters of PBMCs from each donor is added to wells of a 96-well round bottom microtiter plate.

20 Dilutions of the fusion protein test material (50 μ l) is added in triplicate to microtiter wells. Test samples (of the protein of interest) are added for final dilution of 1:4; rhuIL-2 (R&D Systems, Minneapolis, MN, catalog number 202-IL) is added to a final concentration of 1 μ g/ml; anti-CD4 mAb (R&D Systems, clone 34930.11, catalog number MAB379) is added to a final concentration of 10 μ g/ml. Cells are cultured for 7-8 days at 37°C in 5% CO₂, and 1
25 μ C of [³H] thymidine is added to wells for the last 16 hrs of culture. Cells are harvested and thymidine incorporation determined using a Packard TopCount. Data is expressed as the mean and standard deviation of triplicate determinations.

Samples of the fusion protein of interest are screened in separate experiments and compared to the negative control treatment, anti-CD4 mAb, which inhibits proliferation of
30 lymphocytes and the positive control treatment, IL-2 (either as recombinant material or supernatant), which enhances proliferation of lymphocytes.

Example 43: Assays for Protease Activity

The following assay may be used to assess protease activity of an albumin fusion

protein of the invention.

Gelatin and casein zymography are performed essentially as described (Heusen et al., *Anal. Biochem.*, 102:196-202 (1980); Wilson et al., *Journal of Urology*, 149:653-658 (1993)). Samples are run on 10% polyacryamide/0.1% SDS gels containing 1% gelatin
5 orcasein, soaked in 2.5% triton at room temperature for 1 hour, and in 0.1M glycine, pH 8.3 at 37°C 5 to 16 hours. After staining in amido black areas of proteolysis appear as clear areas against the blue-black background. Trypsin (Sigma T8642) is used as a positive control.

Protease activity is also determined by monitoring the cleavage of n-a-benzoyl-L-arginine ethyl ester (BAEE) (Sigma B-4500. Reactions are set up in (25mMNaPO₄, 1mM
10 EDTA, and 1mM BAEE), pH 7.5. Samples are added and the change in adsorbance at 260nm is monitored on the Beckman DU-6 spectrophotometer in the time-drive mode. Trypsin is used as a positive control.

Additional assays based upon the release of acid-soluble peptides from casein or hemoglobin measured as adsorbance at 280 nm or colorimetrically using the Folin method are
15 performed as described in Bergmeyer, et al., *Methods of Enzymatic Analysis*, 5 (1984). Other assays involve the solubilization of chromogenic substrates (Ward, *Applied Science*, 251-317 (1983)).

Example 44: Identifying Serine Protease Substrate Specificity

20 Methods known in the art or described herein may be used to determine the substrate specificity of the albumin fusion proteins of the present invention having serine protease activity. A preferred method of determining substrate specificity is by the use of positional scanning synthetic combinatorial libraries as described in GB 2 324 529 (incorporated herein in its entirety).

25

Example 45: Ligand Binding Assays

The following assay may be used to assess ligand binding activity of an albumin fusion protein of the invention.

Ligand binding assays provide a direct method for ascertaining receptor pharmacology
30 and are adaptable to a high throughput format. The purified ligand for an albumin fusion protein of the invention is radiolabeled to high specific activity (50-2000 Ci/mmol) for binding studies. A determination is then made that the process of radiolabeling does not diminish the activity of the ligand towards the fusion protein. Assay conditions for buffers, ions, pH and other modulators such as nucleotides are optimized to establish a workable signal to noise
35 ratio for both membrane and whole cell polypeptide sources. For these assays, specific polypeptide binding is defined as total associated radioactivity minus the radioactivity

measured in the presence of an excess of unlabeled competing ligand. Where possible, more than one competing ligand is used to define residual nonspecific binding.

Example 46: Functional Assay in Xenopus Oocytes

5 Capped RNA transcripts from linearized plasmid templates encoding an albumin fusion protein of the invention is synthesized in vitro with RNA polymerases in accordance with standard procedures. In vitro transcripts are suspended in water at a final concentration of 0.2 mg/ml. Ovarian lobes are removed from adult female toads, Stage V defolliculated oocytes are obtained, and RNA transcripts (10 ng/oocyte) are injected in a 50 nl bolus using a
10 microinjection apparatus. Two electrode voltage clamps are used to measure the currents from individual *Xenopus oocytes* in response fusion protein and polypeptide agonist exposure. Recordings are made in Ca²⁺ free Barth's medium at room temperature. The *Xenopus* system can be used to screen known ligands and tissue/cell extracts for activating ligands.

15 ***Example 47: Microphysiometric Assays***

Activation of a wide variety of secondary messenger systems results in extrusion of small amounts of acid from a cell. The acid formed is largely as a result of the increased metabolic activity required to fuel the intracellular signaling process. The pH changes in the media surrounding the cell are very small but are detectable by the CYTOSENSOR
20 microphysiometer (Molecular Devices Ltd., Menlo Park, Calif.). The CYTOSENSOR is thus capable of detecting the ability of an albumin fusion protein of the invention to activate secondary messengers that are coupled to an energy utilizing intracellular signaling pathway.

Example 48: Extract/Cell Supernatant Screening

25 A large number of mammalian receptors exist for which there remains, as yet, no cognate activating ligand (agonist). Thus, active ligands for these receptors may not be included within the ligands banks as identified to date. Accordingly, the albumin fusion proteins of the invention can also be functionally screened (using calcium, cAMP, microphysiometer, oocyte electrophysiology, etc., functional screens) against tissue extracts
30 to identify natural ligands for the Therapeutic protein portion and/or albumin protein portion of an albumin fusion protein of the invention. Extracts that produce positive functional responses can be sequentially subfractionated until an activating ligand is isolated and identified.

35 ***Example 49: ATP-binding assay***

The following assay may be used to assess ATP-binding activity of fusion proteins of

the invention.

ATP-binding activity of an albumin fusion protein of the invention may be detected using the ATP-binding assay described in U.S. Patent 5,858,719, which is herein incorporated by reference in its entirety. Briefly, ATP-binding to an albumin fusion protein of the invention is measured via photoaffinity labeling with 8-azido-ATP in a competition assay. Reaction mixtures containing 1 mg/ml of ABC transport protein are incubated with varying concentrations of ATP, or the non-hydrolyzable ATP analog adenylyl-5'-imidodiphosphate for 10 minutes at 4°C. A mixture of 8-azido-ATP (Sigma Chem. Corp., St. Louis, MO.) plus 8-azido-ATP (^{32}P -ATP) (5 mCi/ μmol , ICN, Irvine CA.) is added to a final concentration of 100 μM and 0.5 ml aliquots are placed in the wells of a porcelain spot plate on ice. The plate is irradiated using a short wave 254 nm UV lamp at a distance of 2.5 cm from the plate for two one-minute intervals with a one-minute cooling interval in between. The reaction is stopped by addition of dithiothreitol to a final concentration of 2mM. The incubations are subjected to SDS-PAGE electrophoresis, dried, and autoradiographed. Protein bands corresponding to the albumin fusion proteins of the invention are excised, and the radioactivity quantified. A decrease in radioactivity with increasing ATP or adenylyl-5'-imidodiphosphate provides a measure of ATP affinity to the fusion protein.

Example 50: Phosphorylation Assay

In order to assay for phosphorylation activity of an albumin fusion protein of the invention, a phosphorylation assay as described in U.S. Patent 5,958,405 (which is herein incorporated by reference) is utilized. Briefly, phosphorylation activity may be measured by phosphorylation of a protein substrate using gamma-labeled ^{32}P -ATP and quantitation of the incorporated radioactivity using a gamma radioisotope counter. The fusion protein of the invention is incubated with the protein substrate, ^{32}P -ATP, and a kinase buffer. The ^{32}P incorporated into the substrate is then separated from free ^{32}P -ATP by electrophoresis, and the incorporated ^{32}P is counted and compared to a negative control. Radioactivity counts above the negative control are indicative of phosphorylation activity of the fusion protein.

Example 51: Detection of Phosphorylation Activity (Activation) of an Albumin Fusion Protein of the Invention in the Presence of Polypeptide Ligands

Methods known in the art or described herein may be used to determine the phosphorylation activity of an albumin fusion protein of the invention. A preferred method of

determining phosphorylation activity is by the use of the tyrosine phosphorylation assay as described in US 5,817,471 (incorporated herein by reference).

**Example 52: Identification Of Signal Transduction Proteins That
Interact With An albumin fusion protein Of The Present Invention**

Albumin fusion proteins of the invention may serve as research tools for the identification, characterization and purification of signal transduction pathway proteins or receptor proteins. Briefly, a labeled fusion protein of the invention is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, an albumin fusion protein of the invention is covalently coupled to a chromatography column. Cell-free extract derived from putative target cells, such as carcinoma tissues, is passed over the column, and molecules with appropriate affinity bind to the albumin fusion protein. The protein complex is recovered from the column, dissociated, and the recovered molecule subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

Example 53: IL-6 Bioassay

A variety of assays are known in the art for testing the proliferative effects of an albumin fusion protein of the invention. For example, one such assay is the IL-6 Bioassay as described by Marz *et al.* (*Proc. Natl. Acad. Sci., U.S.A.*, 95:3251-56 (1998), which is herein incorporated by reference). After 68 hrs. at 37°C, the number of viable cells is measured by adding the tetrazolium salt thiazolyl blue (MTT) and incubating for a further 4 hrs. at 37°C. B9 cells are lysed by SDS and optical density is measured at 570 nm. Controls containing IL-6 (positive) and no cytokine (negative) are Briefly, IL-6 dependent B9 murine cells are washed three times in IL-6 free medium and plated at a concentration of 5,000 cells per well in 50 μ l, and 50 μ l of fusion protein of the invention is added. Enhanced proliferation in the test sample(s) (containing an albumin fusion protein of the invention) relative to the negative control is indicative of proliferative effects mediated by the fusion protein.

Example 54: Support of Chicken Embryo Neuron Survival

To test whether sympathetic neuronal cell viability is supported by an albumin fusion protein of the invention, the chicken embryo neuronal survival assay of Senaldi *et al* may be utilized (*Proc. Natl. Acad. Sci., U.S.A.*, 96:11458-63 (1998), which is herein incorporated by reference). Briefly, motor and sympathetic neurons are isolated from chicken embryos,

resuspended in L15 medium (with 10% FCS, glucose, sodium selenite, progesterone, conalbumin, putrescine, and insulin; Life Technologies, Rockville, MD.) and Dulbecco's modified Eagles medium [with 10% FCS, glutamine, penicillin, and 25 mM Hepes buffer (pH 7.2); Life Technologies, Rockville, MD.], respectively, and incubated at 37°C in 5% CO₂ in the presence of different concentrations of the purified fusion protein of the invention, as well as a negative control lacking any cytokine. After 3 days, neuron survival is determined by evaluation of cellular morphology, and through the use of the colorimetric assay of Mosmann (Mosmann, T., *J. Immunol. Methods*, 65:55-63 (1983)). Enhanced neuronal cell viability as compared to the controls lacking cytokine is indicative of the ability of the albumin fusion protein to enhance the survival of neuronal cells.

Example 55: Assay for Phosphatase Activity

The following assay may be used to assess serine/threonine phosphatase (PTPase) activity of an albumin fusion protein of the invention.

In order to assay for serine/threonine phosphatase (PTPase) activity, assays can be utilized which are widely known to those skilled in the art. For example, the serine/threonine phosphatase (PSPase) activity of an albumin fusion protein of the invention may be measured using a PSPase assay kit from New England Biolabs, Inc. Myelin basic protein (MyBP), a substrate for PSPase, is phosphorylated on serine and threonine residues with cAMP-dependent Protein Kinase in the presence of [³²P]ATP. Protein serine/threonine phosphatase activity is then determined by measuring the release of inorganic phosphate from ³²P-labeled MyBP.

Example 56: Interaction of Serine/Threonine Phosphatases with other Proteins

Fusion protein of the invention having serine/threonine phosphatase activity (e.g., as determined in Example 55) are useful, for example, as research tools for the identification, characterization and purification of additional interacting proteins or receptor proteins, or other signal transduction pathway proteins. Briefly, a labeled fusion protein of the invention is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, an albumin fusion protein of the invention is covalently coupled to a chromatography column. Cell-free extract derived from putative target cells, such as neural or liver cells, is passed over the column, and molecules with appropriate affinity bind to the fusion protein. The fusion protein-complex is recovered from the column, dissociated, and the recovered molecule subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate

oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

Example 57: Assaying for Heparanase Activity

There are numerous assays known in the art that may be employed to assay for
5 heparanase activity of an albumin fusion protein of the invention. In one example, heparanase
activity of an albumin fusion protein of the invention, is assayed as described by Vlodavsky
et al., (Vlodavsky et al., Nat. Med., 5:793-802 (1999)). Briefly, cell lysates, conditioned
media, intact cells (1×10^6 cells per 35-mm dish), cell culture supernatant, or purified fusion
10 protein are incubated for 18 hrs at 37°C, pH 6.2-6.6, with ^{35}S -labeled ECM or soluble ECM
derived peak I proteoglycans. The incubation medium is centrifuged and the supernatant is
analyzed by gel filtration on a Sepharose CL-6B column (0.9 x 30 cm). Fractions are eluted
with PBS and their radioactivity is measured. Degradation fragments of heparan sulfate side
chains are eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II). Each experiment is done at
15 least three times. Degradation fragments corresponding to "peak II," as described by
Vlodavsky et al., is indicative of the activity of an albumin fusion protein of the invention in
cleaving heparan sulfate.

Example 58: Immobilization of biomolecules

This example provides a method for the stabilization of an albumin fusion protein of
20 the invention in non-host cell lipid bilayer constructs (see, e.g., Bieri et al., Nature Biotech
17:1105-1108 (1999), hereby incorporated by reference in its entirety herein) which can be
adapted for the study of fusion proteins of the invention in the various functional assays
described above. Briefly, carbohydrate-specific chemistry for biotinylation is used to confine
a biotin tag to an albumin fusion protein of the invention, thus allowing uniform orientation
25 upon immobilization. A 50uM solution of an albumin fusion protein of the invention in
washed membranes is incubated with 20 mM NaIO₄ and 1.5 mg/ml (4mM) BACH or 2
mg/ml (7.5mM) biotin-hydrazide for 1 hr at room temperature (reaction volume, 150ul).
Then the sample is dialyzed (Pierce Slidealizer Cassett, 10 kDa cutoff; Pierce Chemical Co.,
Rockford IL) at 4C first for 5 h, exchanging the buffer after each hour, and finally for 12 h
30 against 500 ml buffer R (0.15 M NaCl, 1 mM MgCl₂, 10 mM sodium phosphate, pH7). Just
before addition into a cuvette, the sample is diluted 1:5 in buffer ROG50 (Buffer R
supplemented with 50 mM octylglucoside).

Example 59: Assays for Metalloproteinase Activity

35 Metalloproteinases are peptide hydrolases which use metal ions, such as Zn^{2+} , as the
catalytic mechanism. Metalloproteinase activity of an albumin fusion protein of the present

invention can be assayed according to methods known in the art. The following exemplary methods are provided:

Proteolysis of alpha-2-macroglobulin

To confirm protease activity, a purified fusion protein of the invention is mixed with the substrate alpha-2-macroglobulin (0.2 unit/ml; Boehringer Mannheim, Germany) in 1x assay buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM CaCl₂, 25 μM ZnCl₂ and 0.05% Brij-35) and incubated at 37°C for 1-5 days. Trypsin is used as positive control. Negative controls contain only alpha-2-macroglobulin in assay buffer. The samples are collected and boiled in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 5-min, then loaded onto 8% SDS-polyacrylamide gel. After electrophoresis the proteins are visualized by silver staining. Proteolysis is evident by the appearance of lower molecular weight bands as compared to the negative control.

Inhibition of alpha-2-macroglobulin proteolysis by inhibitors of metalloproteinases

Known metalloproteinase inhibitors (metal chelators (EDTA, EGTA, AND HgCl₂), peptide metalloproteinase inhibitors (TIMP-1 and TIMP-2), and commercial small molecule MMP inhibitors) may also be used to characterize the proteolytic activity of an albumin fusion protein of the invention. Three synthetic MMP inhibitors that may be used are: MMP inhibitor I, [IC₅₀ = 1.0 μM against MMP-1 and MMP-8; IC₅₀ = 30 μM against MMP-9; IC₅₀ = 150 μM against MMP-3]; MMP-3 (stromelysin-1) inhibitor I [IC₅₀ = 5 μM against MMP-3], and MMP-3 inhibitor II [K_i = 130 nM against MMP-3]; inhibitors available through Calbiochem, catalog # 444250, 444218, and 444225, respectively). Briefly, different concentrations of the small molecule MMP inhibitors are mixed with a purified fusion protein of the invention (50μg/ml) in 22.9 μl of 1x HEPES buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM CaCl₂, 25 μM ZnCl₂ and 0.05%Brij-35) and incubated at room temperature (24 °C) for 2-hr, then 7.1 μl of substrate alpha-2-macroglobulin (0.2 unit/ml) is added and incubated at 37°C for 20-hr. The reactions are stopped by adding 4x sample buffer and boiled immediately for 5 minutes. After SDS-PAGE, the protein bands are visualized by silver stain.

Synthetic Fluorogenic Peptide Substrates Cleavage Assay

The substrate specificity for fusion proteins of the invention with demonstrated metalloproteinase activity may be determined using techniques known in the art, such as using synthetic fluorogenic peptide substrates (purchased from BACHEM Bioscience Inc). Test substrates include, M-1985, M-2225, M-2105, M-2110, and M-2255. The first four are

MMP substrates and the last one is a substrate of tumor necrosis factor- α (TNF- α) converting enzyme (TACE). These substrates are preferably prepared in 1:1 dimethyl sulfoxide (DMSO) and water. The stock solutions are 50-500 μ M. Fluorescent assays are performed by using a Perkin Elmer LS 50B luminescence spectrometer equipped with a constant temperature water bath. The excitation λ is 328 nm and the emission λ is 393 nm. Briefly, the assay is carried out by incubating 176 μ l 1x HEPES buffer (0.2 M NaCl, 10 mM CaCl₂, 0.05% Brij-35 and 50 mM HEPES, pH 7.5) with 4 μ l of substrate solution (50 μ M) at 25 °C for 15 minutes, and then adding 20 μ l of a purified fusion protein of the invention into the assay cuvet. The final concentration of substrate is 1 μ M. Initial hydrolysis rates are monitored for 30-min.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, patent publications, journal articles, abstracts, laboratory manuals, books, or other disclosures) as well as information available through Identifiers specific to databases such as GenBank, GeneSeq, or the CAS Registry, referred to in this application are herein incorporated by reference in their entirety. The specification and sequence listing of each of the following U.S. applications are herein incorporated by reference in their entirety: Application Nos. 60/229,358 filed on April 12, 2000; 60/199,384 filed on April 25, 2000 and 60/256,931 filed on December 21, 2000.

**INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 37, line 14.

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution: American Type Culture Collection

Address of depositary institution (*including postal code and country*)
10801 University Boulevard
Manassas, Virginia 20110-2209
United States of America

Date of deposit

11 April 2001

Accession Number

Unassigned

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*)

This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

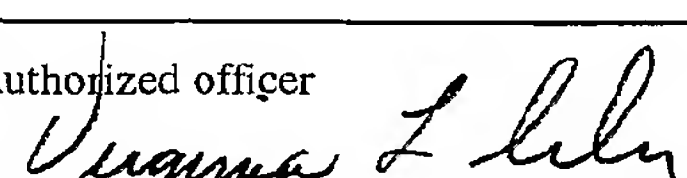
Europe

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

Continued on additional sheets

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the international Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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The applicant requests that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the deposited biological material referred to in the application to an independent expert nominated by the Commissioner, the applicant must, by a written statement, inform the International Bureau accordingly before completion of technical preparations for publication of the international application.

NORWAY

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NETHERLANDS

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Further deposits are identified on an additional sheet ☒

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Address of depositary institution (*including postal code and country*)

10801 University Boulevard
Manassas, Virginia 20110-2209
United States of America

Date of deposit

11 April 2001

Accession Number

Unassigned

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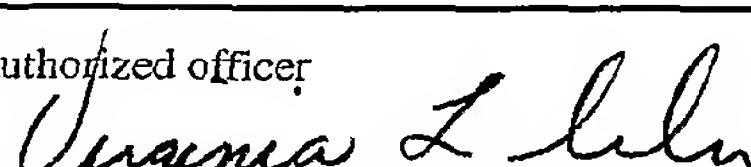
Europe

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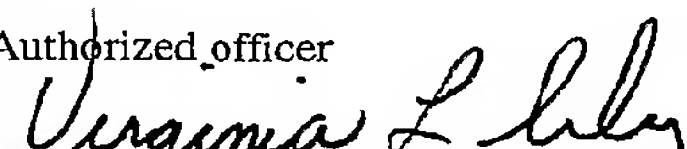
Europe

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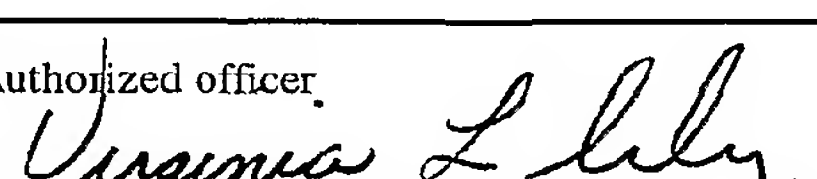
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What is claimed:

1. An albumin fusion protein comprising a Therapeutic protein:X and albumin comprising the amino acid sequence of SEQ ID NO:18.
- 5 2. An albumin fusion protein comprising a Therapeutic protein:X and a fragment or a variant of the amino acid sequence of SEQ ID NO:18, wherein said fragment or variant has albumin activity.
3. The albumin fusion protein of claim 2, wherein said albumin activity is the
10 ability to prolong the shelf life of the Therapeutic protein:X compared to the shelf-life of the Therapeutic protein:X in an unfused state.
4. The albumin fusion protein of claim 2, wherein the fragment or variant comprises the amino acid sequence of amino acids 1-387 of SEQ ID NO:18.
15
5. The albumin fusion protein of any one of claims 1-4, wherein said Therapeutic protein:X comprises IL-2.
6. An albumin fusion protein comprising a fragment or variant of a Therapeutic
20 protein:X, and albumin comprising the amino acid sequence of SEQ ID NO:18, wherein said fragment or variant has a biological activity of the Therapeutic protein:X.
7. The albumin fusion protein of claim 6, wherein said Therapeutic protein:X comprises IL-2, and wherein said fragment or variant has T cell proliferative activity or T
25 cell activation activity.
8. The albumin fusion protein of any one of claims 1-4 or 6, wherein said Therapeutic protein:X, or fragment or variant thereof, comprises a protein selected from the

group consisting of:

- (a) calcitonin;
- (b) growth hormone releasing factor;
- (c) IL-2 fusion protein;
- 5 (d) insulin-like growth factor-1;
- (e) interferon beta; and
- (f) parathyroid hormone.

9. The albumin fusion protein of any one of claims 1-8, wherein the
10 Therapeutic protein:X, or fragment or variant thereof, is fused to the N-terminus of albumin,
or the N-terminus of the fragment or variant of albumin.

10. The albumin fusion protein of any one of claims 1-8, wherein the
Therapeutic protein:X, or fragment or variant thereof, is fused to the C-terminus of albumin,
15 or the C-terminus of the fragment or variant of albumin.

11. The albumin fusion protein of any one of claims 1-8, wherein the
Therapeutic protein:X, or fragment or variant thereof, is fused to the N- terminus and C-
terminus of albumin, or the N-terminus and the C-terminus of the fragment or variant of
20 albumin.

12. The albumin fusion protein of any one of claims 1-8, which comprises a first
Therapeutic protein:X, or fragment or variant thereof, and a second Therapeutic protein:X,
or fragment or variant thereof, wherein said first Therapeutic protein:X, or fragment or
25 variant thereof, is different from said second Therapeutic protein:X, or fragment or variant
thereof.

13. The albumin fusion protein of any one of claims 1-11, wherein the

Therapeutic protein:X, or fragment or variant thereof, is separated from the albumin or the fragment or variant of albumin by a linker.

14. The albumin fusion protein of any one of claims 1-11, wherein the albumin
5 fusion protein has the following formula:

R1-L-R2; R2-L-R1; or R1-L-R2-L-R1,

wherein R1 is Therapeutic protein:X, or fragment or variant thereof, L is a peptide
linker, and R2 is albumin comprising the amino acid sequence of SEQ ID NO:18 or fragment
or variant of albumin.

10

15. The albumin fusion protein of any one of claims 1-14, wherein the shelf-life
of the albumin fusion protein is greater than the shelf-life of the Therapeutic protein:X, or
fragment or variant thereof, in an unfused state.

15 16. The albumin fusion protein of any one of claims 1-14, wherein the in vitro
biological activity of the Therapeutic protein:X, or fragment or variant thereof, fused to
albumin, or fragment or variant thereof, is greater than the in vitro biological activity of the
Therapeutic protein:X, or fragment or variant thereof, in an unfused state.

20 17. The albumin fusion protein of any one of claims 1-14, wherein the in vivo
biological activity of the Therapeutic protein:X, or fragment or variant thereof, fused to
albumin, or fragment or variant thereof, is greater than the in vivo biological activity of the
Therapeutic protein:X, or fragment or variant thereof, in an unfused state.

25 18. An albumin fusion protein comprising a Therapeutic protein:X, or fragment
or variant thereof, inserted into an albumin comprising the amino acid sequence of SEQ ID
NO:18 or fragment or variant thereof.

19. An albumin fusion protein comprising a Therapeutic protein:X, or fragment or variant thereof, inserted into an albumin comprising an amino acid sequence selected from the group consisting of:

- (a) amino acids 54 to 61 of SEQ ID NO:18;
- 5 (b) amino acids 76 to 89 of SEQ ID NO:18;
- (c) amino acids 92 to 100 of SEQ ID NO:18;
- (d) amino acids 170 to 176 of SEQ ID NO:18;
- (e) amino acids 247 to 252 of SEQ ID NO:18;
- (f) amino acids 266 to 277 of SEQ ID NO:18;
- 10 (g) amino acids 280 to 288 of SEQ ID NO:18;
- (h) amino acids 362 to 368 of SEQ ID NO:18;
- (i) amino acids 439 to 447 of SEQ ID NO:18;
- (j) amino acids 462 to 475 of SEQ ID NO:18;
- (k) amino acids 478 to 486 of SEQ ID NO:18; and
- 15 (l) amino acids 560 to 566 of SEQ ID NO:18.

20. The albumin fusion protein of claims 18 or 19, wherein said albumin fusion protein comprises a portion of albumin sufficient to prolong the shelf-life of the Therapeutic protein:X, or fragment or variant thereof, as compared to the shelf-life of the Therapeutic protein:X, or fragment or variant thereof, in an unfused state.

21. The albumin fusion protein of claims 18 or 19, wherein said albumin fusion protein comprises a portion of albumin sufficient to prolong the in vitro biological activity of the Therapeutic protein:X, or fragment or variant thereof, fused to albumin as compared to the in vitro biological activity of the Therapeutic protein:X, or fragment or variant thereof, in an unfused state.

22. The albumin fusion protein of claims 18 or 19 wherein said albumin fusion

protein comprises a portion of albumin sufficient to prolong the in vivo biological activity of the Therapeutic protein:X, or fragment or variant thereof, fused to albumin compared to the in vivo biological activity of the Therapeutic protein:X, or fragment or variant thereof, in an unfused state.

5

23. The albumin fusion protein of any one of claims 1-22, which is non-glycosylated.

10

24. The albumin fusion protein of any one of claims 1-22, which is expressed in yeast.

25. The albumin fusion protein of claim 24, wherein the yeast is glycosylation deficient.

15

26. The albumin fusion protein of claim 24 wherein the yeast is glycosylation and protease deficient.

27. The albumin fusion protein of any one of claims 1-22, which is expressed by a mammalian cell.

20

28. The albumin fusion protein of any one of claims 1-22, wherein the albumin fusion protein is expressed by a mammalian cell in culture.

25

29. The albumin fusion protein of any one of claims 1-22, wherein the albumin fusion protein further comprises a secretion leader sequence.

30. A composition comprising the albumin fusion protein of any one of claims 1-29 and a pharmaceutically acceptable carrier.

31. A kit comprising the composition of claim 30.

32. A method of treating a disease or disorder in a patient, comprising the step of
5 administering the albumin fusion protein of any one of claims 1-29.

33. The method of claim 32, wherein the disease or disorder comprises
indication:Y.

10 34. The method of claim 33, wherein the Therapeutic protein:X, or fragment or
variant thereof, comprises IL-2 and the disease or disorder is selected from the group
consisting of: metastatic renal cell carcinoma; metastatic melanoma; malignant melanoma;
renal cell carcinoma; HIV infection; inflammatory bowel disorder; Kaposi's sarcoma;
leukaemia; multiple sclerosis; rheumatoid arthritis; transplant rejection; type 1 diabetes
15 mellitus; lung cancer; acute myeloid leukaemia; hepatitis C; non-hodgkin's lymphoma; and
ovarian cancer.

35. A method of treating a patient with a disease or disorder that is modulated by
Therapeutic protein:X, comprising the step of administering an effective amount of the
20 albumin fusion protein of any one of claims 1-29.

36. The method of claim 35, wherein the disease or disorder is indication:Y.

37. The method of claim 36, wherein the Therapeutic protein:X, or fragment or
25 variant thereof, is IL-2 and the disease or disorder is selected from the group consisting of:
metastatic renal cell carcinoma; metastatic melanoma; malignant melanoma; renal cell
carcinoma; HIV infection; inflammatory bowel disorder; Kaposi's sarcoma; leukaemia;
multiple sclerosis; rheumatoid arthritis; transplant rejection; type 1 diabetes mellitus; lung

cancer; acute myeloid leukaemia; hepatitis C; non-hodgkin's lymphoma; and ovarian cancer.

38. A method of extending the shelf life of Therapeutic protein:X comprising the step of fusing the Therapeutic protein:X, or fragment or variant thereof, to albumin or a
5 fragment or variant thereof of albumin sufficient to extend the shelf-life of the Therapeutic protein:X, or fragment or variant thereof, compared to the shelf-life of the Therapeutic protein:X, or fragment or variant thereof, in an unfused state.

39. A nucleic acid molecule comprising a polynucleotide sequence encoding the
10 albumin fusion protein of any one of claims 1-29.

40. A vector comprising the nucleic acid molecule of claim 39.

41. A host cell comprising the nucleic acid molecule of claim 39.

15

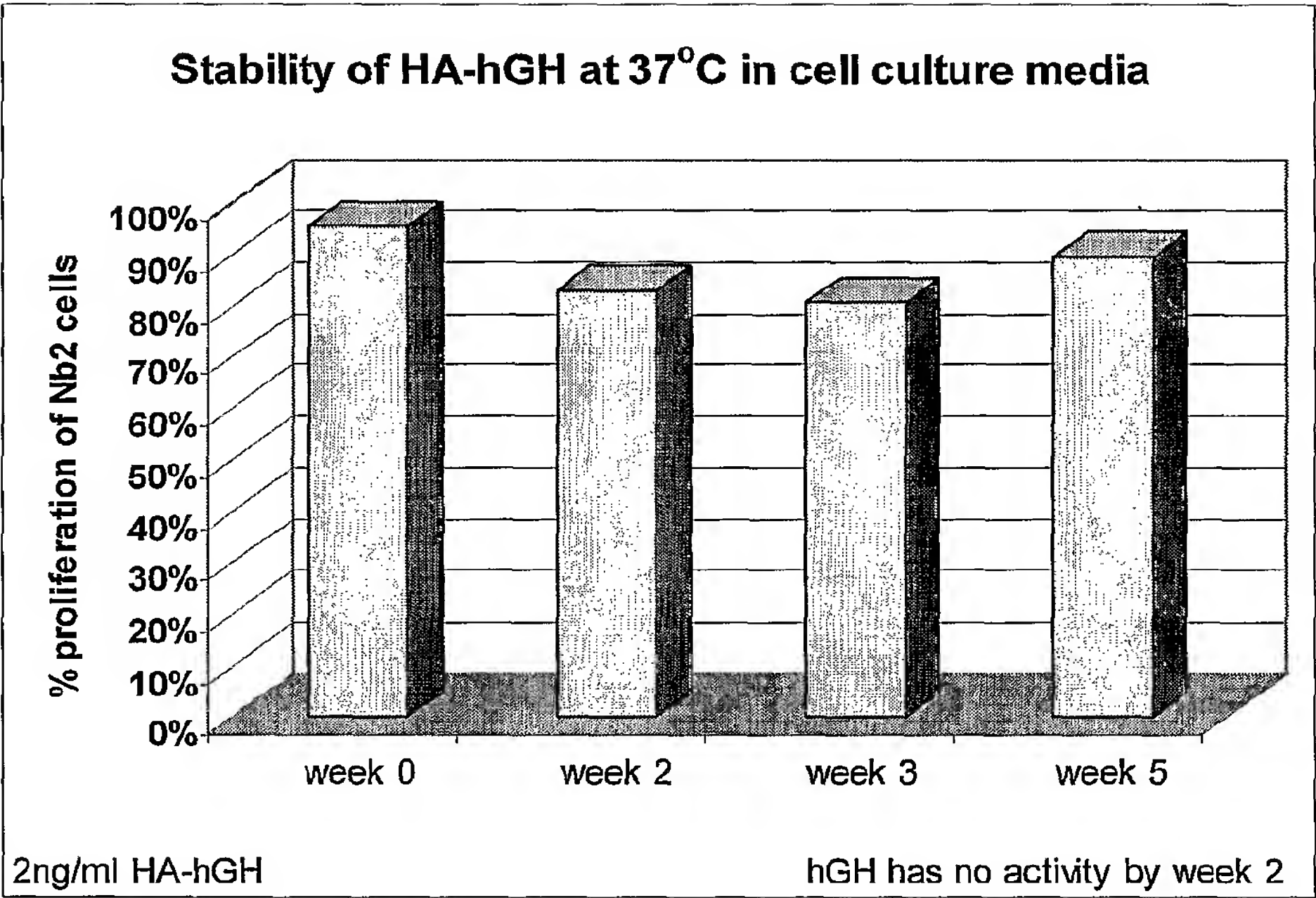


Figure 1

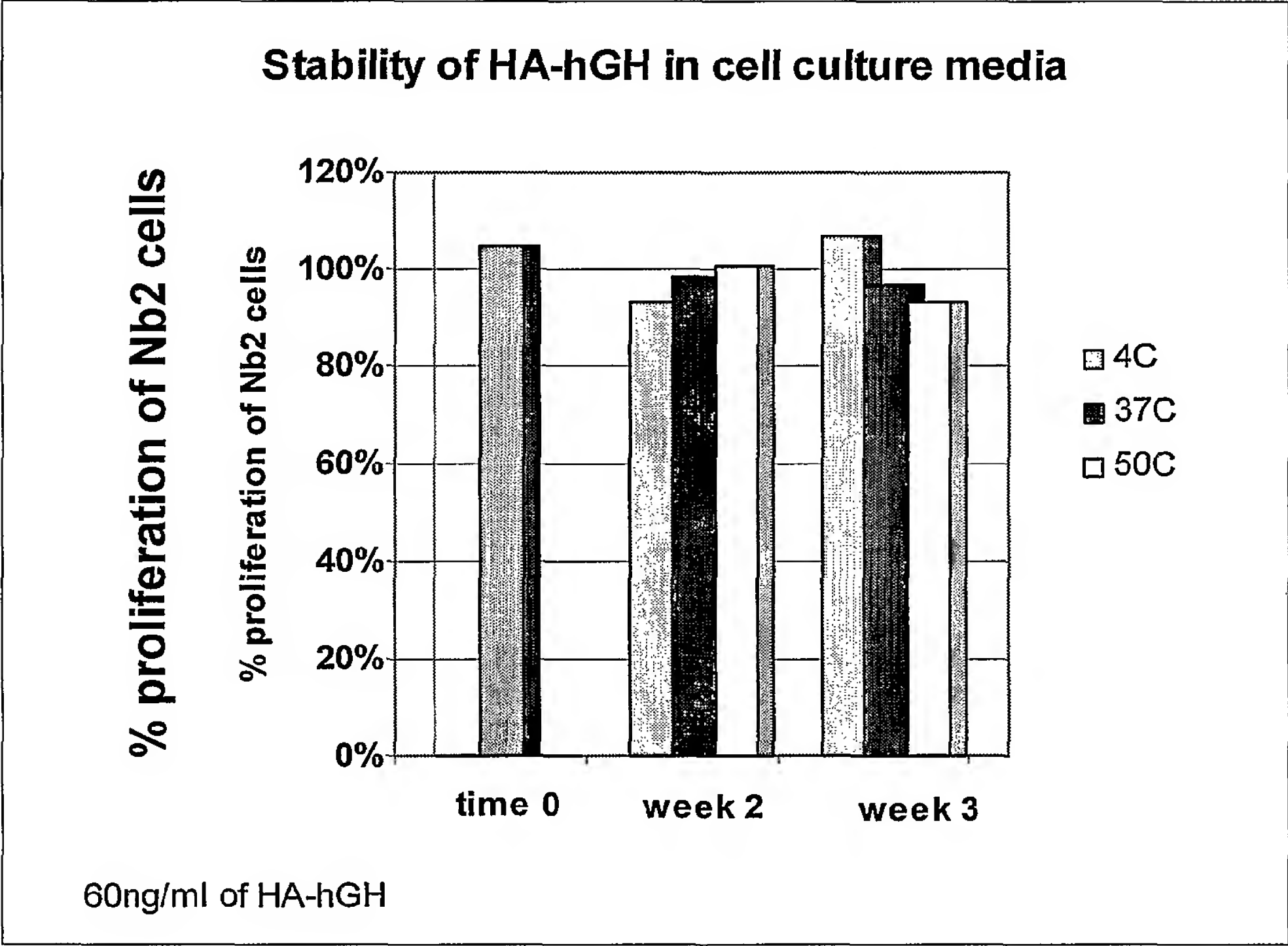


Figure 2

3/20

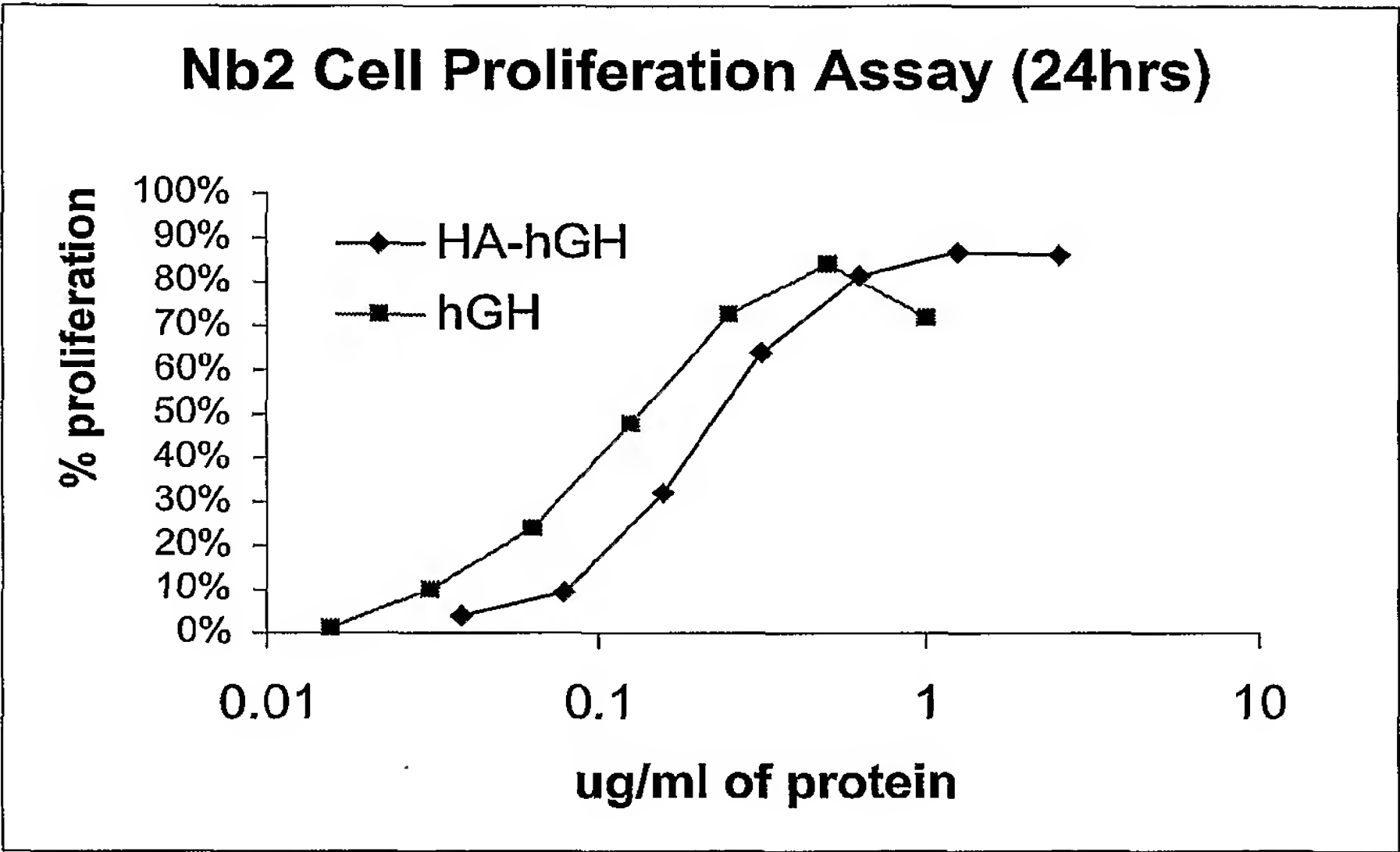


Figure 3A

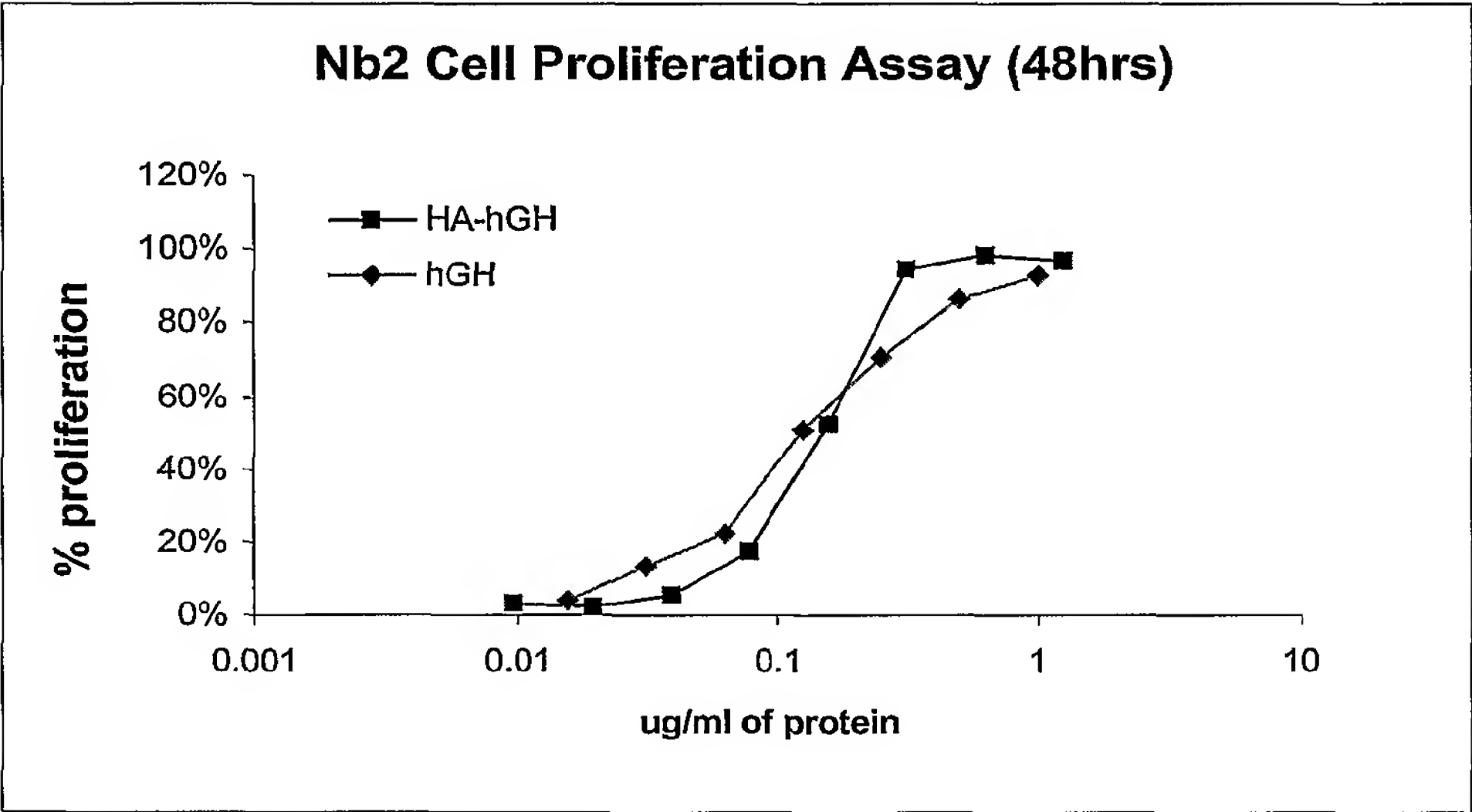


Figure 3B

4/20

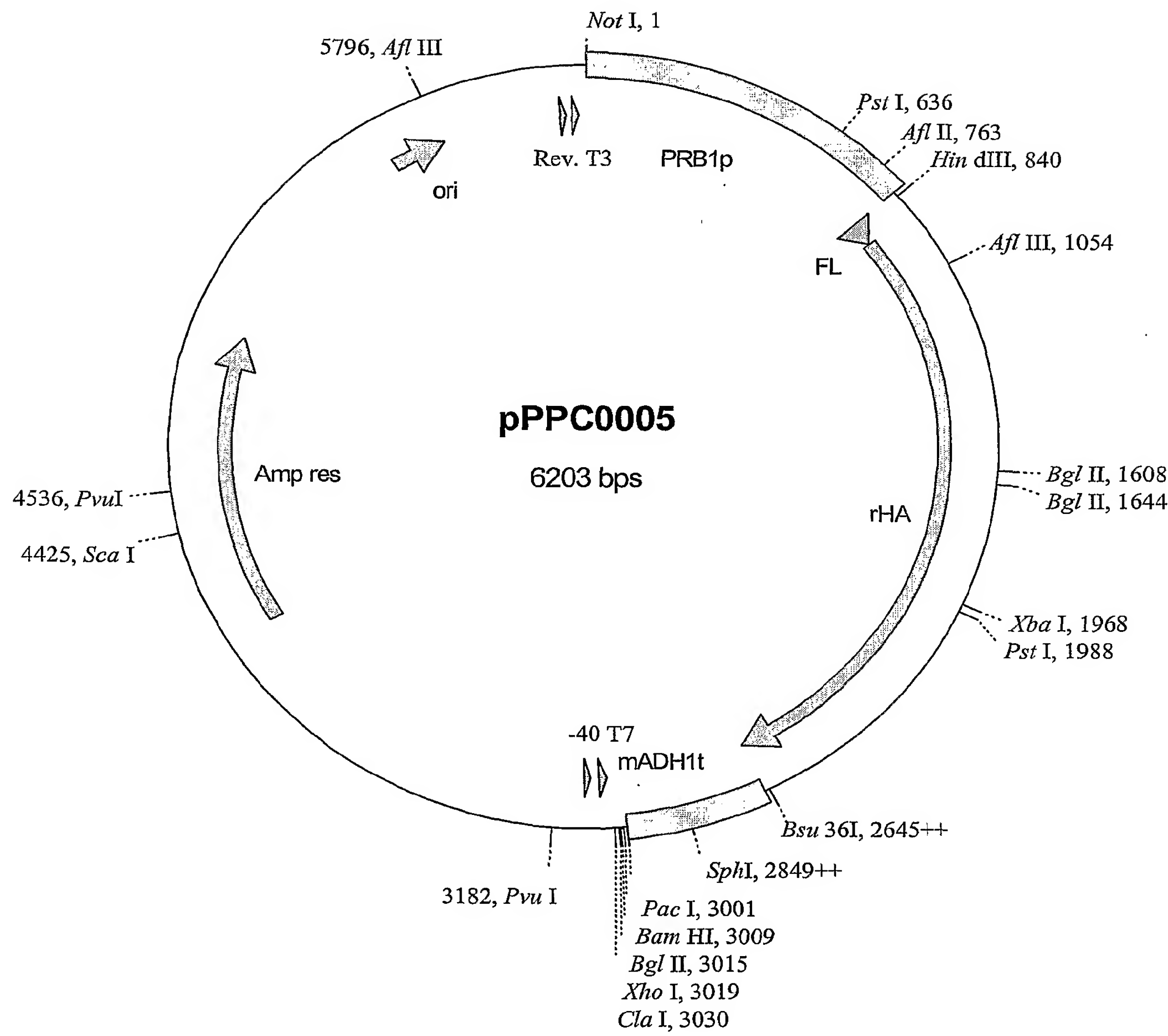


Figure 4

5/20

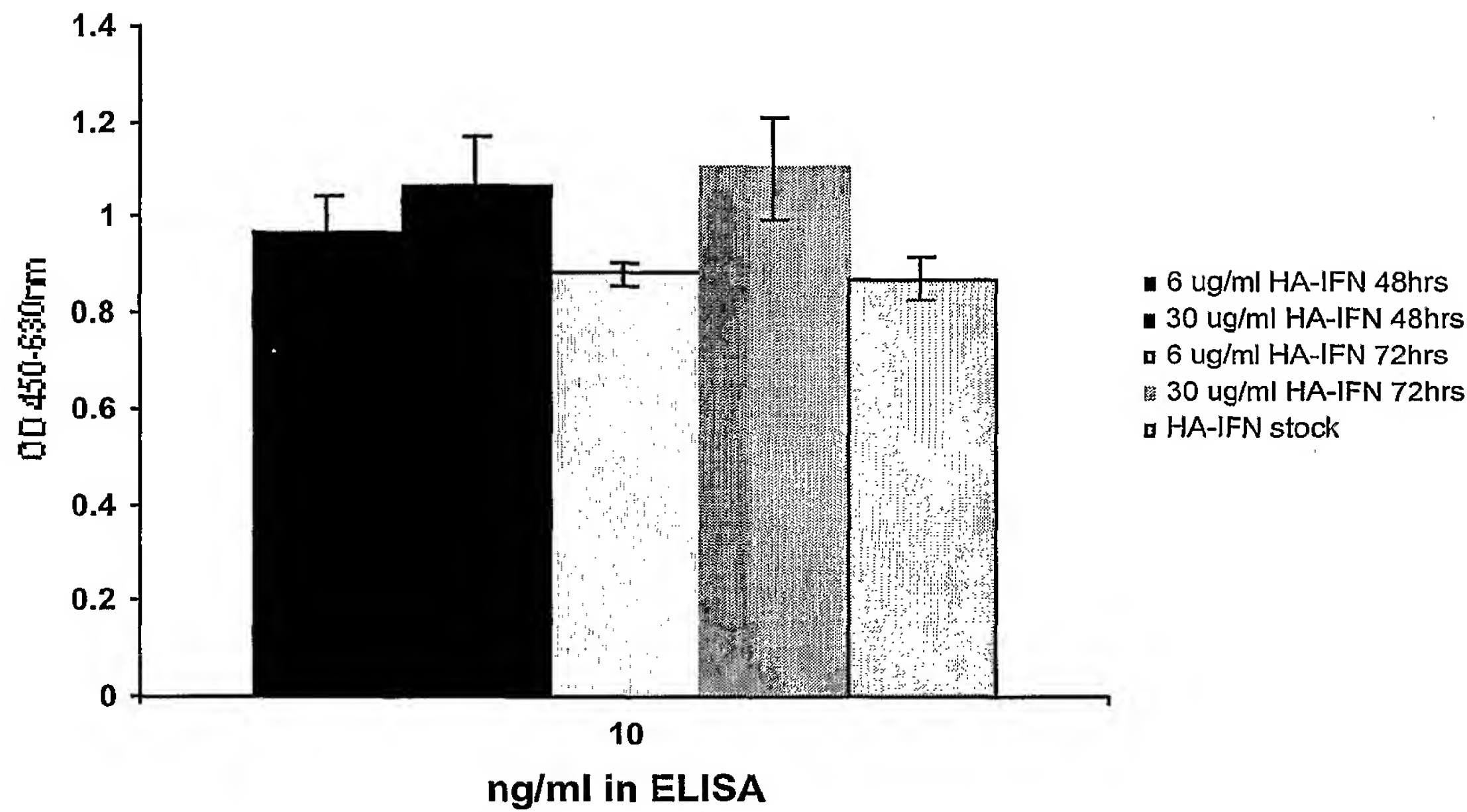
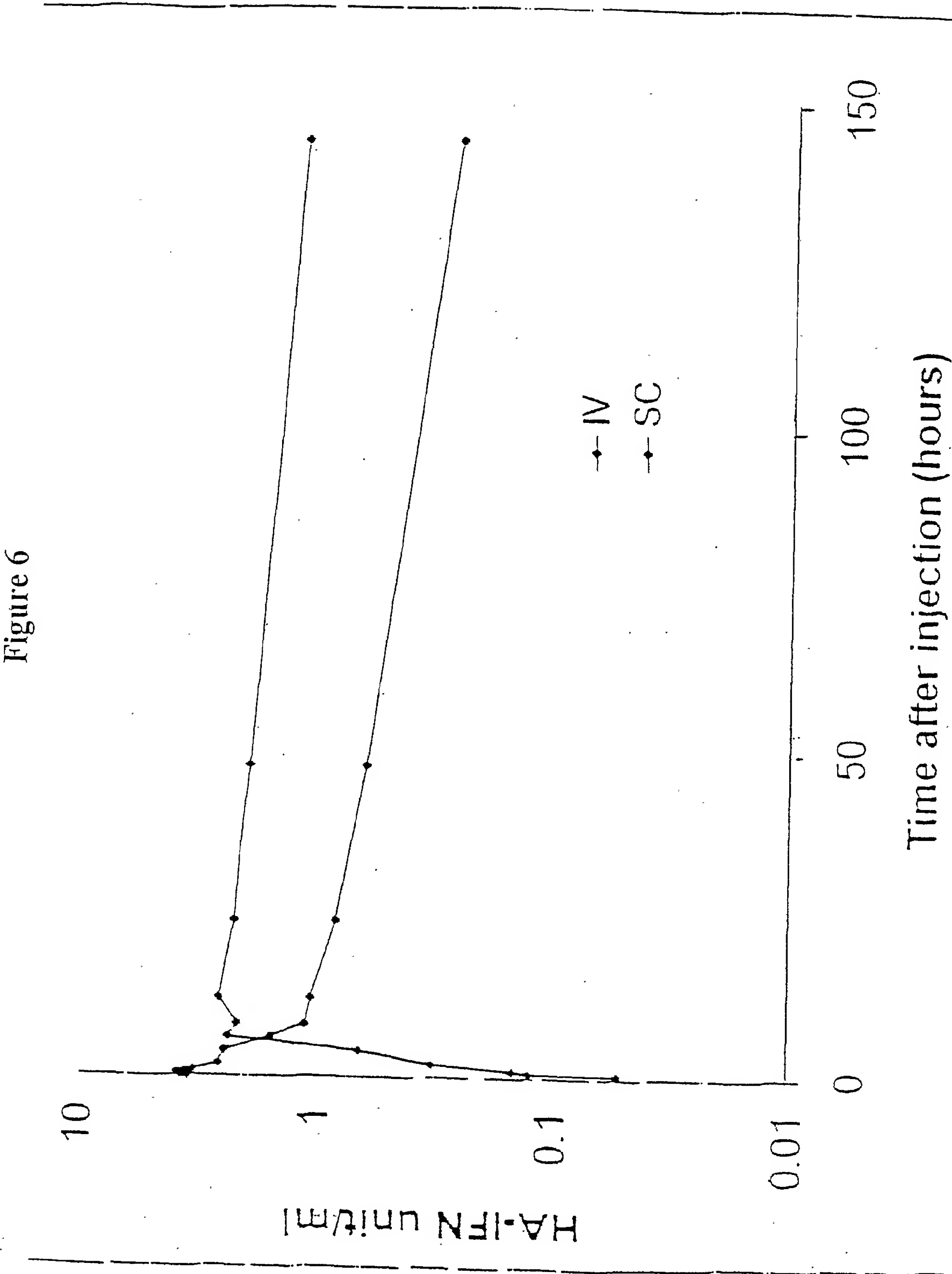


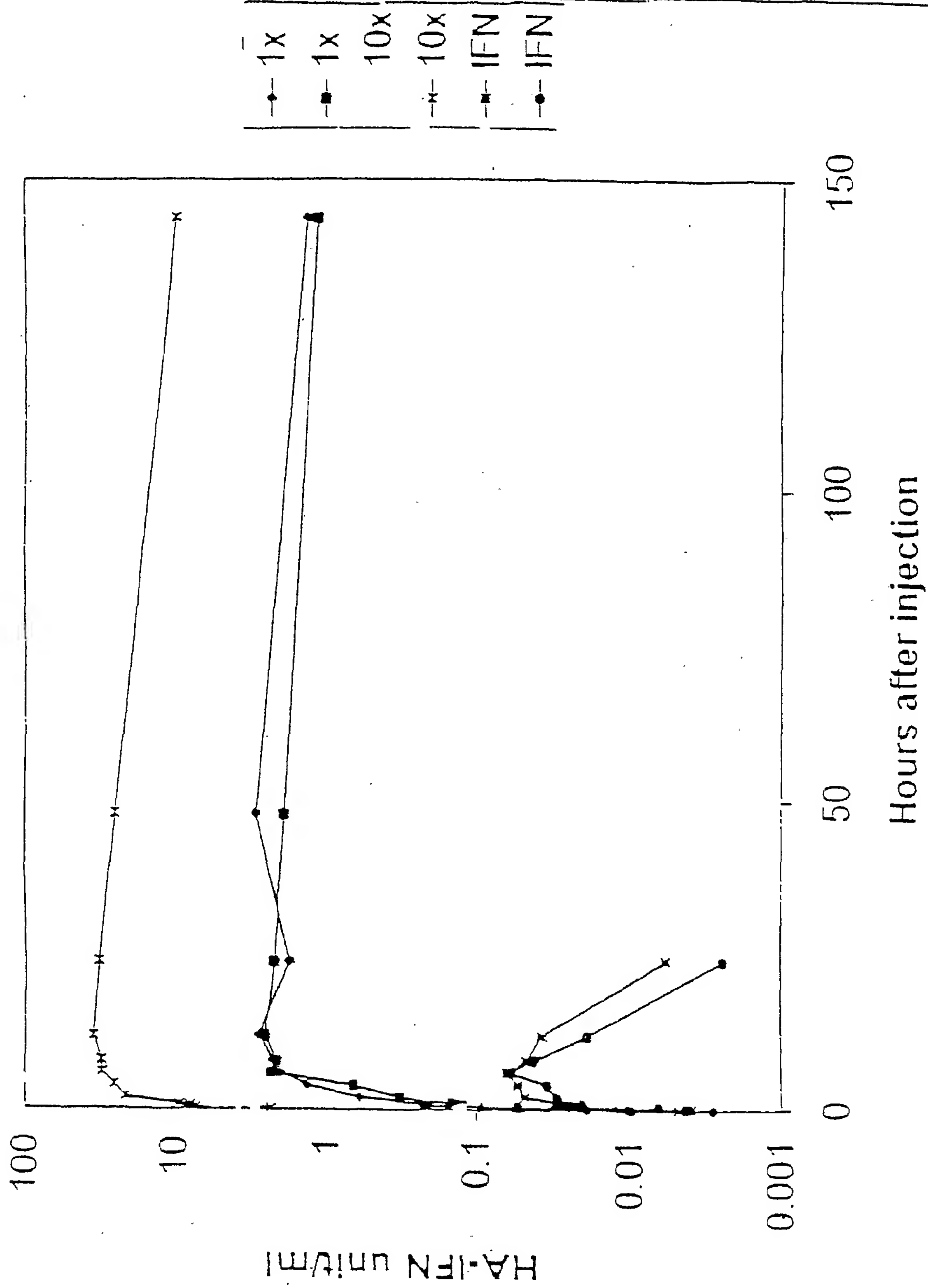
Figure 5

6/20



7/20

Figure 7



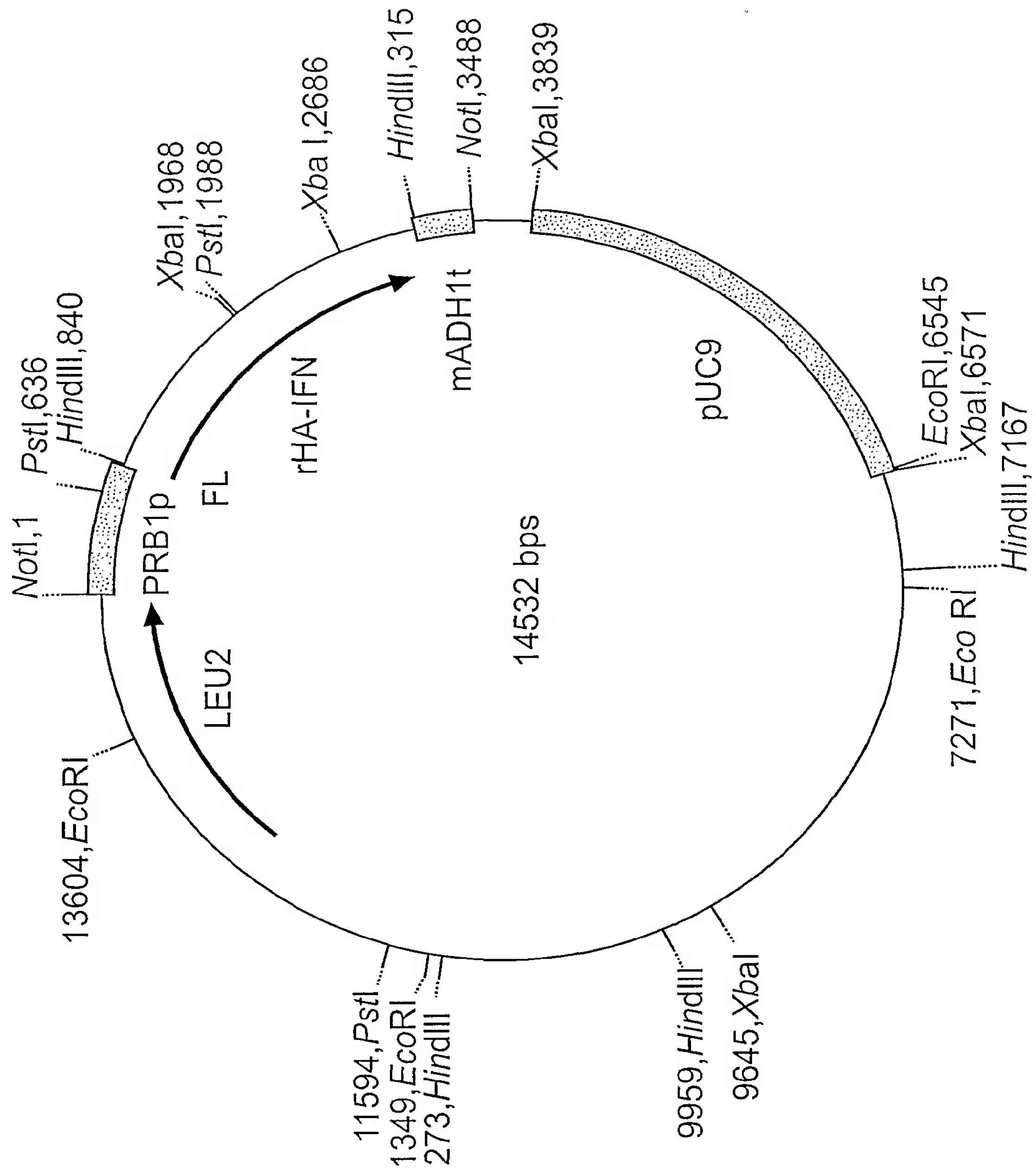


FIG. 8

9/20

Localisation of 'Loops' based on the HA Crystal Structure
which could be used for Mutation/Insertion

1	DAHKSEVAHR	FKDLGEENFK	ALVLI AFAQY	LQCCPFEDHV	KLVNEVTEFA
	HHHHH	HHH	HHH	HHHHH	HHHHHHHHHH
	<div style="display: flex; justify-content: space-around;"> I II III </div>				
51	KTCV <u>ADESAE</u>	NCDKSLHTLF	GDKLCTVATL	RETYGEMADC	CAKOE <u>PERNE</u>
	HHHHH	HHHHH	HHHHH	HHHH	H HHHH
101	CFLQHKDDNP	NLPRLVRPEV	DVMCTAFHDN	EETFLKKYLY	EIARRHPYFY
	HHHH	H	HHHHHHHH	HHHHHHHHHH	HHHHH
	IV				
151	APELLFFAKR	YKAAFTECCO	<u>AADKAA</u> CLLP	KLDEL RDEGK	ASSAKQRLKC
	HHHHHHHHHH	HHHHHHHHHH	HHHHH	HHHEHHHHHH	HHHHHHHHHH
	V				
201	ASLQKFGERA	FKAWAVARLS	QRFPKAEFAE	VSKLVTDLTk	VHTECC <u>HGDL</u>
	HHHHH	HH	HHHHHHHHHH	HH	HHH HHHHHHHHHH HHHHHH HH
	<div style="display: flex; justify-content: space-around;"> VI VII </div>				
251	LECADDRADL	AKYICE <u>NODS</u>	<u>ISSKLKECCE</u>	KPLLEKSHCI	AEVENDEMPPA
	HHHHHHHHHH	HHHHH	HHHHH	HHHHHHH	H
301	DLPSLAADFV	ESKDVCKNYA	EAKDVFLGMF	LYEYARRHPD	YSVVL LRLA
	HHHH	HHHHHH	HHHHHHH	HHHHHH	HHHHHHHH
	VIII				
351	KTYETTLEKC	<u>CAAADPHECY</u>	AKVFDEFKPL	VEEPQNLIKQ	NCELFEOQLGE
	HHHHHHHHHH	HH	H	HHHHH	HHHHHHHHHH HHHHHH
	IX				
401	YKFQNALLVR	YTKKVPQVST	PTLVEVSRNL	GKVGSKCCKH	<u>PEAKRMPCAE</u>
	HHHHHHHHHH	HHHH	H	HHHHHHHHHH	HHH HHHHHHHH
	<div style="display: flex; justify-content: space-around;"> X XI </div>				
451	DYLSVVLNQL	<u>CVLHEKTPVS</u>	<u>DRVTKCCTES</u>	<u>LVNRRPPCFSA</u>	LEVDETYVPK
	HHHHHHHHHH	HHHHH	HHHHHHHHH	HHHHHHHH	
501	EFNAETFTFH	ADICTLSEKE	RQIKKQTALV	ELVKHKPKAT	KEQLKAVMDD
		HHH	HHH	HHHHHMEHHH	HHH HHHHHHHH
	XII				
551	FAAFVEKCCK	<u>ADDKET</u> CFAE	EGKKLVAAASQ	AALGL	
	HHHHHHHH	HHHH	HHHHHHHHHH	HH	

Loop

I Val154-Asn61
 II Thr76-Asp89
 III Ala92-Glu100
 IV Gln170-Ala176
 V His247-Glu252
 VI Glu266-Glu277

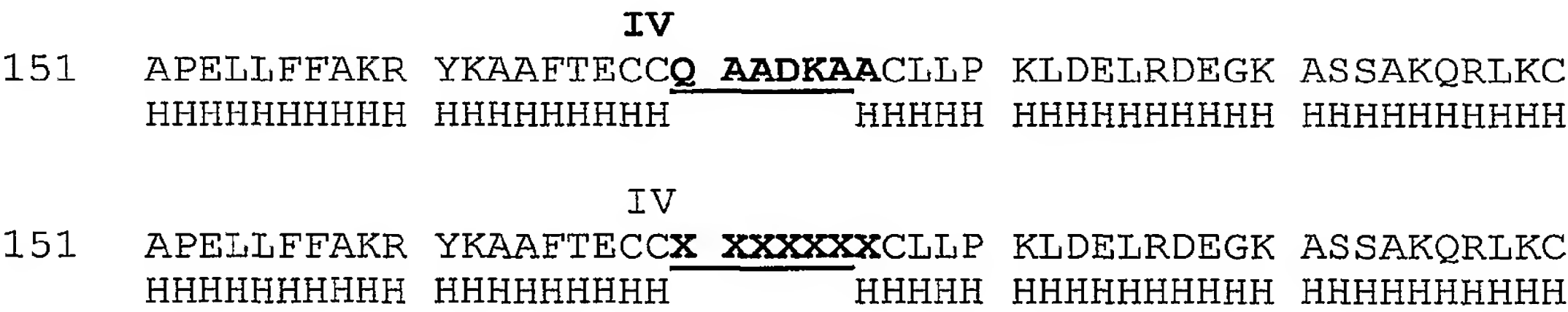
Loop

VII Glu280-His288
 VIII Ala362-Glu368
 IX Lys439-Pro447
 X Val462-Lys475
 XI Thr478-Pro486
 XII Lys560-Thr566

Figure 9

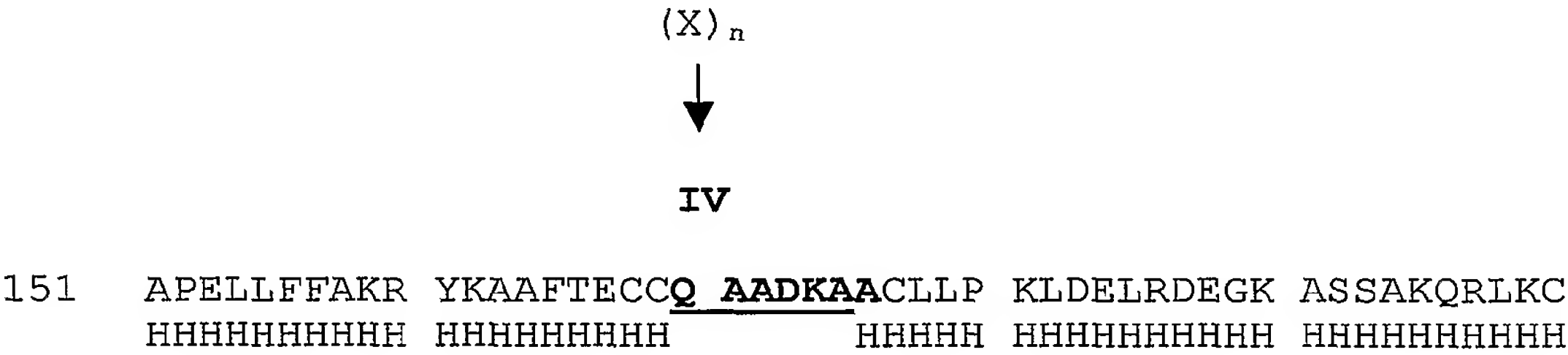
Examples of Modifications to Loop IV

a. Randomisation of Loop IV.



X represents the mutation of the natural amino acid to any other amino acid. One, more or all of the amino acids can be changed in this manner. This figure indicates all the residues have been changed.

b. Insertion (or replacement) of Randomised sequence into Loop IV.



The insertion can be at any point on the loop and the length a length where n would typically be 6, 8, 12, 20 or 25.

Figure 10

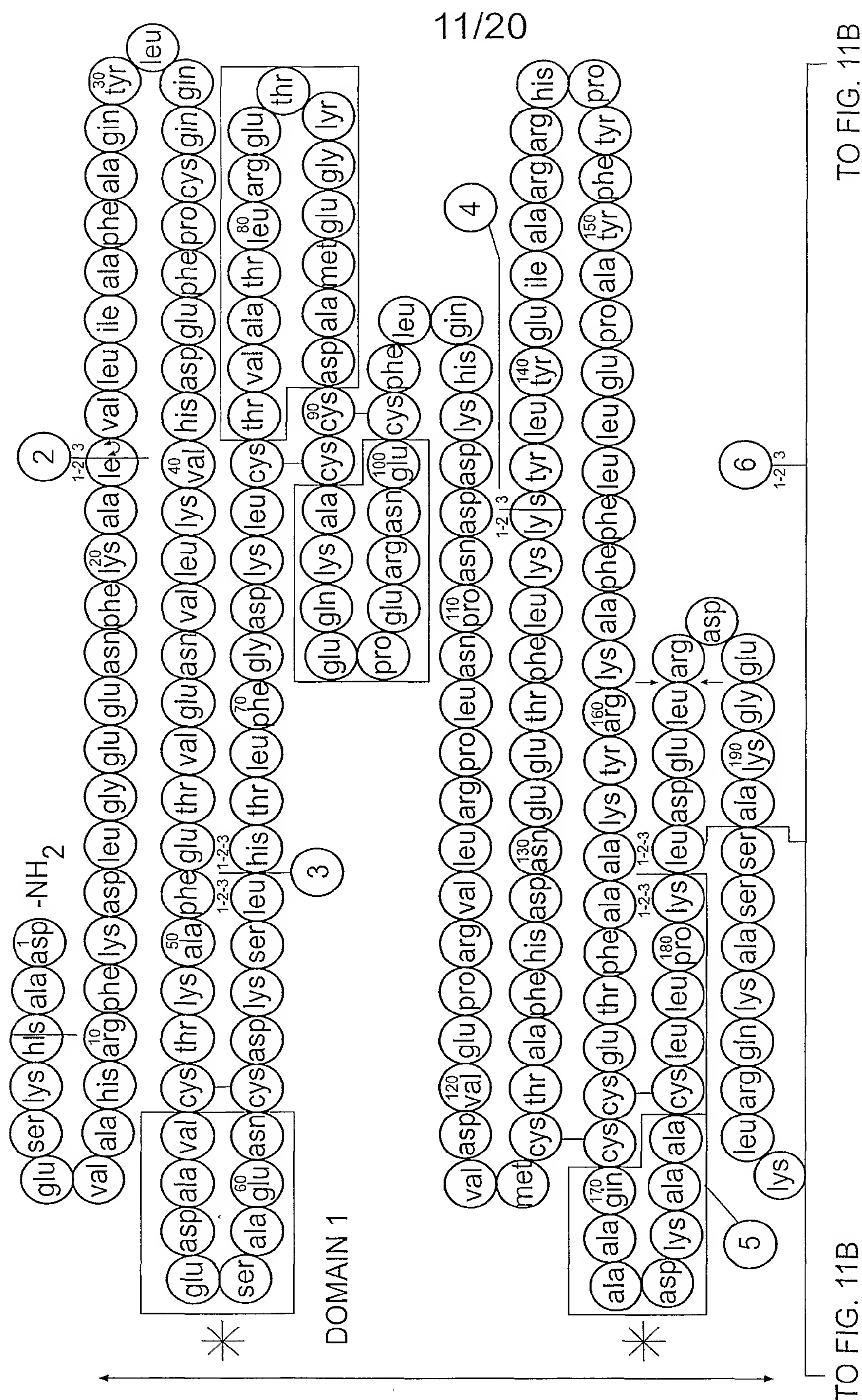
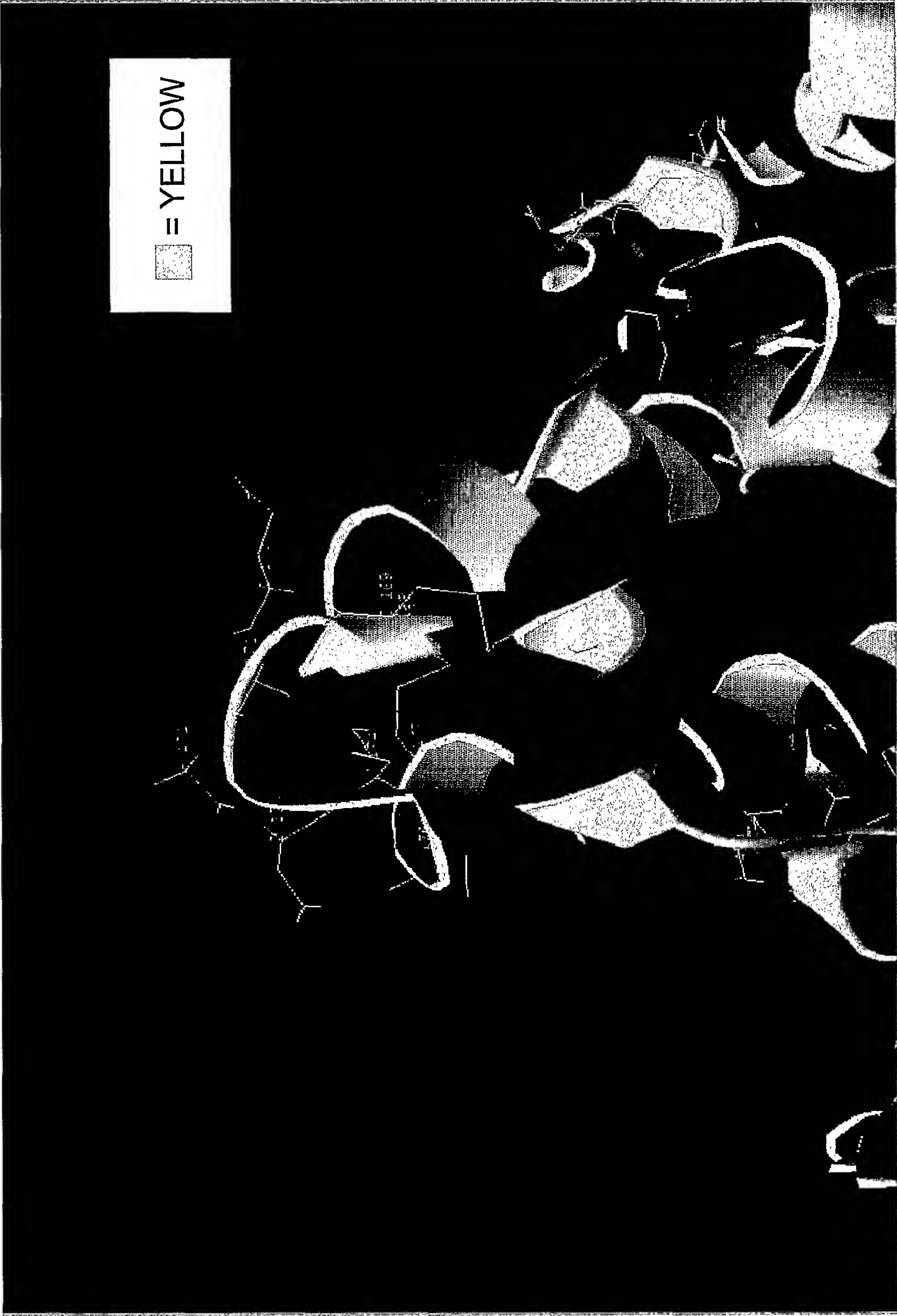


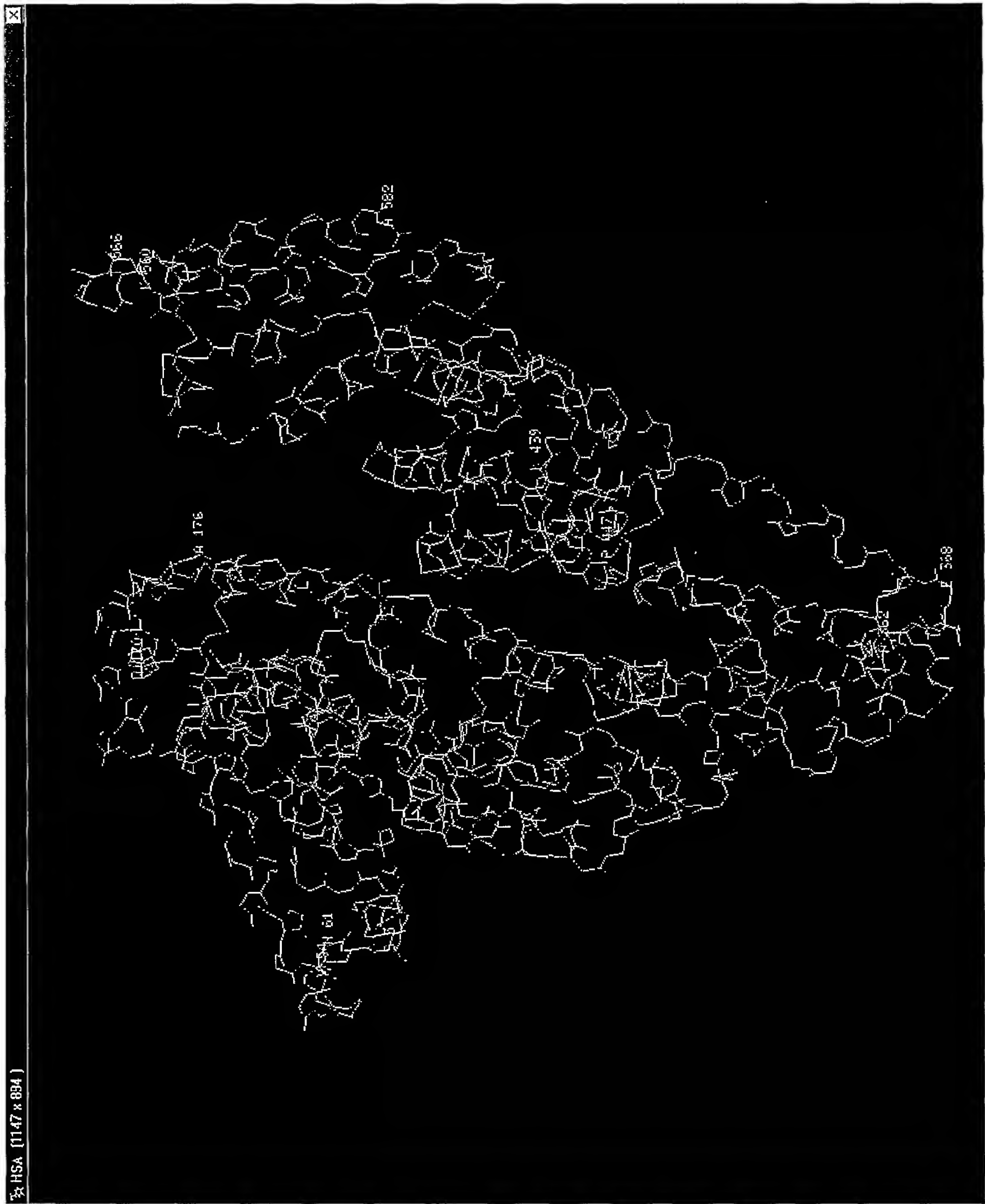
FIG. 11A



 = YELLOW

DISULFIDE BONDS SHOWN IN YELLOW

FIG. 12:
LOOP IV GLU170-A176



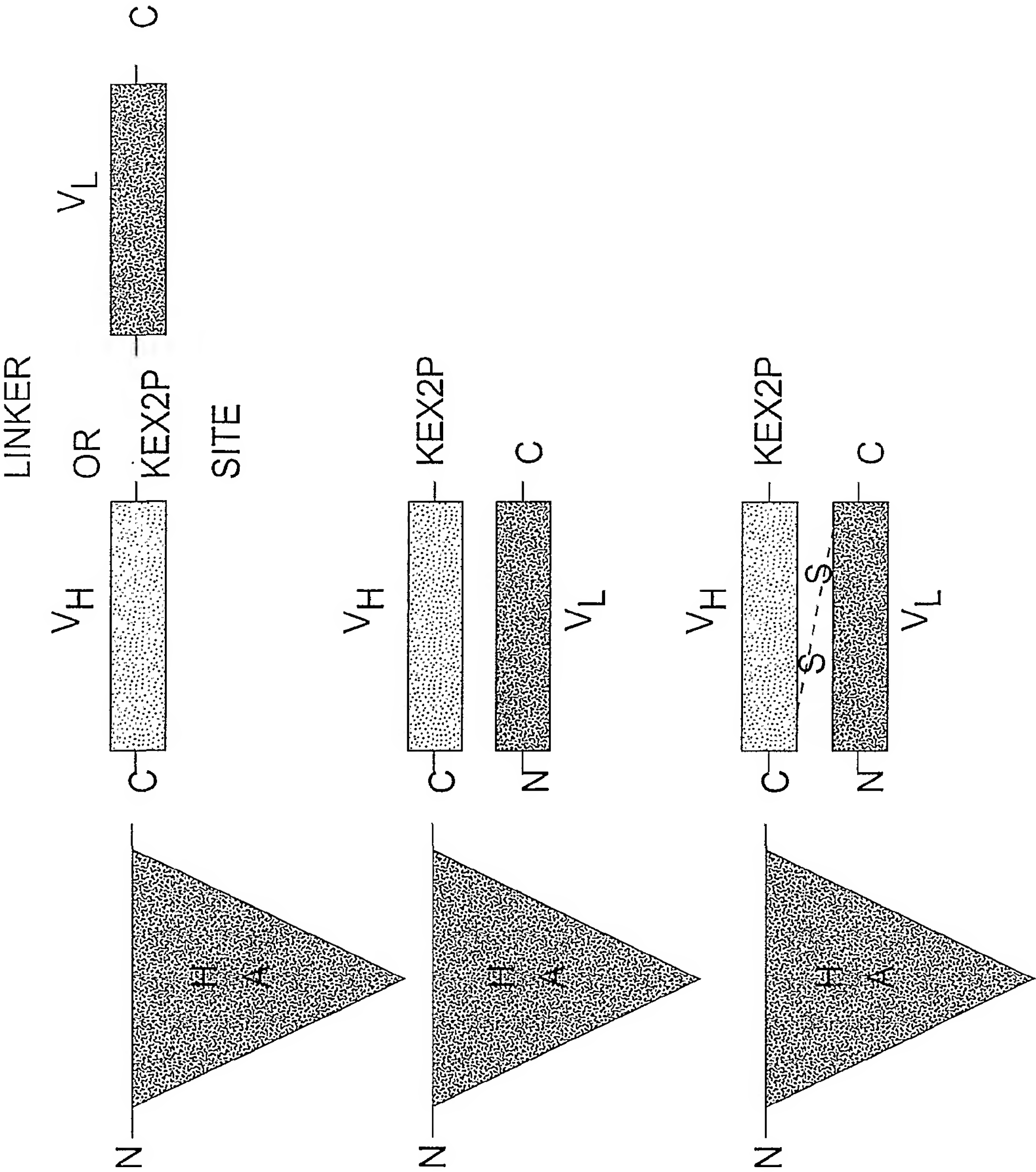


FIG. 14

1 GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA GAT TTG GGA GAA AAT TTC AAA 60
1 D A H K S E V A H R F K D L G E E N F K 20

61 GCC TTG GTG TTG ATT GCC TTT GCT CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA 120
21 A L V L I A A F A Q Y L Q Q C P F E D H V 40

121 AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA 180
41 K L V N E V T E F A K T C V A D E S A E 60

181 AAT TGT GAC AAA TCA CTT CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA GTT GCA ACT CTT 240
61 N C D K S L H T L F G D K L C T V A T L 80

241 CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA AAA CAA GAA CCT GAG AGA AAT GAA 300
81 R E T Y G E M A D C C A K Q E P E R N E 100

301 TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT 360
101 C F L Q H K D N P N L P R L V R P E V 120

361 GAT GTG ATG TGC ACT GCT TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT 420
121 D V M C T A F H D N E E T F L K K Y L Y 140

421 GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG 480
141 E I A R R R H P Y F Y A P E L L F F A K R 160

Figure 15A

481	TAT	AAA	GCT	GCT	TTT	ACA	GAA	TGT	TGC	CAA	GCT	GCT	GAT	AAA	GCT	GCC	TGC	CTG	TTG	CCA	540
161	Y	K	A	A	F	T	E	C	C	Q	A	A	D	K	A	A	C	L	L	P	180
541	AAG	CTC	GAT	GAA	CTT	CGG	GAT	GAA	GGG	AAG	GCT	TCG	TCT	GCC	AAA	CAG	AGA	CTC	AAA	TGT	600
181	K	L	D	E	L	R	D	E	G	K	A	S	S	A	K	Q	R	L	K	C	200
601	GCC	AGT	CTC	CAA	AAA	TTT	GGA	GAA	AGA	GCT	TTC	AAA	GCA	TGG	GCA	GTG	GCT	CGC	CTG	AGC	660
201	A	S	L	Q	K	F	G	E	R	A	F	K	A	W	A	V	A	R	L	S	220
661	CAG	AGA	TTT	CCC	AAA	GCT	GAG	TTT	GCA	GAA	GTT	TCC	AAG	TTA	GTG	ACA	GAT	CTT	ACC	AAA	720
221	Q	R	F	P	K	A	E	F	A	E	V	S	K	L	V	T	D	L	T	K	240
721	GTC	CAC	ACG	GAA	TGC	TGC	CAT	GGA	GAT	CTG	CTT	GAA	TGT	GCT	GAT	GAC	AGG	GCG	GAC	CTT	780
241	V	H	T	E	C	C	H	G	D	L	L	E	C	A	D	D	R	A	D	L	260
781	GCC	AAG	TAT	ATC	TGT	GAA	AAT	CAG	GAT	TCG	ATC	TCC	AGT	AAA	CTG	AAG	GAA	TGC	TGT	GAA	840
261	A	K	Y	I	C	E	N	Q	D	S	I	S	S	K	L	K	E	C	C	E	280
841	AAA	CCT	CTG	TTG	GAA	AAA	TCC	CAC	TGC	ATT	GCC	GAA	GTG	GAA	AAT	GAT	GAG	ATG	CCT	GCT	900
281	K	P	L	L	E	K	S	H	C	I	A	E	V	E	N	D	E	M	P	A	300
901	GAC	TTG	CCT	TCA	TTA	GCT	GCT	GAT	TTT	GTT	GAA	AGT	AAG	GAT	GTT	TGC	AAA	AAC	TAT	GCT	960
301	D	L	P	S	L	A	A	D	F	V	E	S	K	D	V	C	K	N	Y	A	320

Figure 15B

Figure 15C